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BIOLOGICAL STUDIES OF *DERMOCYSTIDIUM MARINUM*, A FUNGOUS PARASITE OF OYSTERS

by

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INTRODUCTION

1. Historical Review of D. marinum and Related Forms.

In 1950 Mackin, Owen, and Collier published the description of a recently discovered protistan parasite, Dermocystidium marinum, in the commercial oyster, Crassostrea virginica (Gmelin). This parasite is now known to be widely distributed in oysters on the South Atlantic and Gulf Coasts of the United States. In spite of its wide distribution and common occurrence in great abundance, the discovery of this important parasite was probably greatly delayed for two reasons: (1) careful histological examination was necessary for its detection; (2) more attention was paid to environmental conditions than to histopathological conditions of the oyster in the study of oyster mortality.

A brief résumé of the genus Dermocystidium was presented by Mackin et al. (l. c.), and they also briefly discussed the known species of this genus and their hosts. This genus was first erected by Pérez, in 1907, under the name Dermocystis. Since the name was preoccupied by Dermocystis Stafford, 1905, the new name Dermocystidium was proposed for the genus by Pérez in 1908.

The occurrence of D. marinum in an estuarine host sets it apart from all the other known species of the genus, which are parasites of fresh-water hosts. Nine species of the genus Dermocystidium have been described: two from amphibians, five from fish, and two from invertebrate hosts. The type species D. pusula (Pérez,

1907) has been reported from the skin of various salamanders (Triturus marmoratus, T. cristatus, T. alpestris, and T. palmatus) and from the obstetrical toad (Alytes obstetricans). D. ranae Guyénot and Naville, 1922, occurs in the skin of Rana temporaria and R. esculenta. The two species which parasitize amphibians are known only from continental Europe, whereas the five species that have been found in fish are more widely distributed, being known from Europe, North America, and Asia. D. branchialis Léger, 1914, from the gills of brown trout (Trutta fario); D. vejdovskyi Jirovec, 1939, from the gills of pike (Esox lucius); and D. percae Reichenbach-Klinke, 1950, from the skin of a perch (Perca fluviatilis) constitute the European species. A North American form, D. salmonis Davis, 1947, has been reported from the gills of a single adult chinook salmon (Oncorhynchus tschawytscha) from the Sacramento River, California. In Japan, D. koi Hoshina and Sahara, 1950, occurs in the skin and in the muscle tissue of the carp (Cyprinus carpio). Another European species, D. daphniae Jirovec, 1939, found in the body cavity of Daphnia magna was described and illustrated by Rühberg (1933) although he did not assign a name to it. This form was later placed in the genus Dermocystidium by Jirovec (1939). Later Weiser (1943) removed D. daphniae from this genus and erected the genus Lymphocystidium for it. He suggested that it may be a microsporidian. Thus D. marinum is the only parasite of invertebrates still retained in the genus. How-

ever, it too will probably be removed from the genus Dermocystidium in the future.

D. marinum differs from all other species assigned to the genus in that the organisms do not occur in definite cysts. Since there exists no clear-cut generic diagnosis, there is considerable doubt as to whether D. marinum is congeneric with the other species of Dermocystidium. Probably this question cannot be answered until something is known of the life cycle of the various species. The nature of the species comprising the genus Dermocystidium as well as those of other genera of cyst-forming parasites of vertebrates, appearing superficially at least to be related, has been a matter of much speculation. Most authors have considered the majority of the species to be lower fungi while some species have been related to the protozoa (Sporozoa) by a few authors. Practically nothing is known of the life cycles of these forms.

Among the well-established genera, Mackin et al. (l. c.) considered Dermocystidium to be the most closely related to Blastocystis Alexeieff (1911). Léger (1914) related Dermocystidium to the order Haplosporidia (Sporozoa) as did Davis (1947) who indicated that this order was so ill-defined that many unrelated forms may be placed in it. The observation of flagellated zoospores (de Beauchamp, 1914) attributed to Dermocystidium from the skin of Triturus, and the reported confirmation (Grassé, 1926 and 1927) led Ciferri (1932) to place this genus in the family Olpidaceae (Phycomycetes). Broz

and Privora (1952) pointed out that de Beauchamp's observations were actually made on Dermomycoides armoriacus Poisson, 1936, and D. beccarii Granata, 1919. These parasites of European urodeles (Triturus palmatus and T. vulgaris) were placed in the family Chytridiaceae (Phycomycetes) by Poisson (1937).

Recently, Goodchild (1953) erected a new genus Histocystidium for a new species of fungoid, cyst-forming parasites occurring subcutaneously in five bullfrogs (Rana catesbeiana) collected from freshwater ponds near Woods Hole, Massachusetts, in 1949. He states that this new species, Histocystidium ranae, has not been encountered since the original collections although collections were made in the same locality in 1950 and 1951. In addition to the description of the genus Histocystidium, this author gave an excellent review and discussion of the species in the five other genera which are generally considered together in this probably heterogenous group of cyst-forming parasites of vertebrates. He also discussed the life history possibilities of some of the species in this group and compared Histocystidium with the five possibly related genera.

Two of these genera, Dermocystidium and Dermomycoides, have been mentioned above. Dermosporidium, which is closely related to Dermocystidium, contains three species: A. hylarum Carini, 1940, from the skin of the tree frog (Hyla rubra) from Brazil; D. granulosum Broz and Privora, 1952, from the skin of Rana temporaria from Czechoslavakia; and D. trutta Weiser, 1949, from the gills

of the brown trout (Trutta fario) from Europe. The species in this genus contain numerous inclusion bodies or "vacuoplasts" in the mature spore instead of a single one as in Dermocystidium. Another genus of this group, Heptosphaera, contains a single species, H. molgarum Gambier, 1924, which is found in the liver of salamanders of the genus Triturus. The fifth genus of this peculiar assemblage of parasites to be considered is Rhinosporidium which contains a human parasite, R. seeberi (Wernicke, 1903), and closely related forms infecting horses, mules, and cows. This fungous parasite, which produces cysts (sporangia) in infected tissues, causes polypoid growths of the nose, eyes, ears, penis, etc.

Cultural studies carried out in the course of this investigation definitely establish the fungous nature of D. marinum and support Mackin's later suggestion (1951a) that D. marinum may be related to certain mycotic disease-producing organisms of such genera as Cryptococcus, Blastomyces, Coccidioides, Rhinosporidium, and others---all of which produce a yeast-like cell in the host. Sparrow (1953) is of the opinion that some of the forms observed in culture as well as other aspects suggest relationship of D. marinum to the order Entomophthorales (Phycomycetes) which contains principally parasites of insects. Pérez (1907) also considered that D. pusula may be related to this group of fungi.

The typical morphological appearances of D. marinum as seen in stained histological sections of oyster tissue were described and

illustrated by Mackin et al. (l. c.). The small spherical organisms as seen in or among host tissue cells normally measure from 2 μ to 20 μ, occasionally even 30 μ in diameter, the average being approximately 10 μ. The presence of a large polymorphic refringent inclusion body or "vacuoplast" within a single, very large, partially eccentric vacuole which occupies the greater part of the body of the organism is the chief distinguishing characteristic of D. marinum, and this feature is mainly responsible for its assignment to the genus Dermocystidium.

Reproduction of the parasites within the host takes place by multiple fission (Mackin et al. (l. c.). This method of reproduction has not been reported as occurring in any of the other species of Dermocystidium. The number of the daughter cells produced by a single mother cell may vary greatly, from 3 to 4, to as many as 25 or 30. The size of the mother cells is within the range of the single organisms.

2. Statement of the Problem and its Significance.

Since 1936 extensive oyster mortalities (ranging up to 95 per cent) occurring during the summer and early fall and associated with high temperature and high salinity, have been reported frequently from the Gulf Coast area, especially on cultivated beds in Louisiana (McConnell, 1936, 1940, 1942, and 1950; Owen, 1950, 1953a, and 1953b; Owen et al., 1951; Mackin, 1951b; Butler, 1952;

Hopkins, 1952; Collier, in press; and Sieling, 1953). Much evidence has been accumulated which indicates that D. marinum is associated with this mortality. Mackin et al. (l. c.) considered that low temperature and low salinity evidently retarded development of the infestation. Mackin (1951a) reported that massive infections develop in oysters under conditions of high temperature and high salinity.

Investigations conducted in Virginia (Hewatt and Andrews, 1953, and in press; Andrews and Hewatt, 1953) revealed a periodicity of mortality in oysters maintained in trays at Gloucester Point that is similar to that found in Louisiana, although the mortality was somewhat less. The annual mortalities in the trays averaged 28 per cent, with 24 per cent occurring during the warmer months (June through October). These authors reported that 228 (76 per cent) of the dying oysters were checked for D. marinum and 90 per cent were infected; and 72 per cent of the infections were classified as heavy. They also found that checks of 398 live oysters from various stations during August, 1952, showed only 25 per cent infection and 7 per cent were heavily infected.

The results obtained by the above authors closely paralleled those obtained earlier by Mackin (1951b) with regard to distribution of the relative intensity of infection in "gapers" (oysters that are unable to maintain closure of valves shortly before death) and live oysters. In this study, conducted in Barataria Bay, Louisiana, he found that the intensity of infection was much greater in "gapers"

than in survivors. Of 198 "gapers" examined during the study, 94 per cent were found to be infected; 86 per cent were classified as heavy. On the contrary, the distribution of the relative intensity of infection in 214 survivors examined during the same study was largely reversed. This group showed infections of only 42 per cent, with 3 per cent heavily infected and 27 per cent lightly infected.

The findings of the above studies clearly indicate that there is an association between Dermocystidium infection and mortality in oysters, although a direct cause and effect relationship has not been demonstrated. On epizootiological grounds alone, the evidence incriminating D. marinum as a cause of much of the oyster mortality is very strong. This evidence consists of: (1) direct correlation of oyster mortality and Dermocystidium infection; (2) direct correlation of both oyster mortality and Dermocystidium infection with the temperature and the salinity of the water; and (3) similar seasonal periodicity of oyster mortality and Dermocystidium infection (high in warmer months and greatly reduced in colder months).

The present investigation was undertaken to attempt to learn something about the biology and life cycle of D. marinum as a preliminary to studies on the transmission and pathogenicity of this parasite. Evidence obtained by means of controlled pathogenicity studies in conjunction with histopathological evidence (Mackin, 1951a) and epizootiological evidence already available would enable one to evaluate more accurately the role of this parasite as a mortality

agent. Furthermore, knowledge of the biology and life cycle of the parasite may suggest control measures or indicate the environmental conditions under which injury from infection might be held to a minimum. Moreover, other methods of combating D. marinum may be evolved such as the development of resistant strains of oysters either experimentally or by natural selection.

The first step in working out the life cycle of this parasite was an effort to develop a technique for growing it in artificial culture.

CULTURAL STUDIES

1. Development of Culture Technique for Diagnosis.

Preliminary attempts to culture D. marinum consisted of inoculating various artificial media with small amounts of pericardial fluid from infected oysters. These initial attempts, details of which have been previously presented (Ray, 1952a), were unsuccessful. The failure to culture the parasite by means of some of the conventional methods suggested the possibility of this organism being an obligate parasite; in this case living tissue might be required for its development. During early attempts to grow oyster tissue in tissue culture, it was discovered that D. marinum in oyster tissue becomes greatly enlarged after a relatively short period of incubation in a nutrient medium. This observation led to the development of an easy and accurate method for the diagnosis of Dermocystidium infections in oysters.

Since the details of this culture technique have been presented elsewhere (Ray, 1952a, 1952b, and 1952c), only a summary of the details in addition to subsequent modifications will be presented here. The technique consists of: (1) planting small pieces of oyster tissue in tubes containing fluid thioglycollate medium rehydrated with distilled water containing 20 grams of NaCl per liter, and fortified with penicillin and streptomycin (250 to 500 units of each per ml. of medium) to suppress bacterial growth; (2) incubating the tubes for a minimum of 48 hours at room temperature (18° to 30° C.);

(3) staining the tissue with iodine solution; and (4) microscopically examining the tissues for cultured parasites. The cultured parasites become conspicuously enlarged into cystlike bodies (Fig. 1) usually reaching a maximum diameter of approximately 70 to 125 μ after 48 to 72 hours incubation; the walls of the cultured parasites stain a characteristic blue when treated with iodine. The uncultured organisms do not react in this manner. The intensity of the blue reaction increases with incubation until the stained organisms appear as opaque, blue-black bodies. On a few occasions some of the organisms have been observed to reach a diameter of nearly 300 μ after incubation of about a week. No appreciable increase in size is usually observed after 72 to 96 hours of incubation, although the walls generally become thicker after prolonged incubation. The cultured parasites are extremely stable; they show no evidence of disintegration after months of incubation, even when the medium is contaminated with molds and bacteria. The parasites average somewhat larger in light and moderate infections. Sometimes a great majority of the parasites fail to enlarge appreciably in spite of the fact that the incubation temperature is at the optimum and bacterial growth is adequately retarded to permit normal growth. This failure to enlarge in a normal manner is encountered most frequently in heavily infected tissues, and possibly is due to exhaustion of some necessary substances either in the tissues or in the medium. The heart, rectum, and pieces of gill and mantle (about 5 x 10 mm.)

should be incubated if an extremely thorough diagnosis of light infections is desired, but for survey purposes the rectum alone will suffice.

For examination the tissue is placed on a slide and flooded with iodine solution (1:5 aqueous dilution of Lugol's stock solution). Then the tissue is shredded with needles to facilitate greater penetration of the iodine and is allowed to stand approximately five minutes before a cover slip is applied. It is essential that some fragments of the tissue be stained brown at least at the edges; if not adequately stained, more iodine solution is added. The excess solution is removed with strips of absorbent paper toweling, while the tissue is flattened as much as possible by pressure on the cover slip. In heavily infected tissues so treated, a blue color visible macroscopically is imparted to the tissue. When examined microscopically (30 to 100X magnification) in stained preparations, the majority of the parasites appear in varying shades of blue; some appear green, greenish-brown, and brown.

The reliability of this technique for indicating the incidence of D. marinum has been well established by extensive use by several oyster biologists. It has also been employed to estimate intensity of infection since no discernible increase in the number of enlarged forms has been noticed even after prolonged incubation of thioglycollate cultures. Occasionally some of the cultured parasites germinate, producing short hyphae or germ tubes which produce terminal,

spherical, budlike processes (Figs. 2 and 3). This results in some increase in number, but it is so limited that it does not influence the estimation of the intensity of infections. These germinating forms have been encountered much more frequently and abundantly during the colder months, and they have been observed almost entirely in heavily infected oysters. In most cases careful microscopic search is necessary to find these forms; occasionally large numbers have been encountered. The hyphal and budding forms have been observed more frequently in thioglycollate medium rehydrated with distilled water than in medium rehydrated with sea water or distilled water containing 20 grams of NaCl per liter.

The above observations indicate that the technique may be reliably used to estimate intensity of infection unless appreciable multiplication occurs prior to enlargement of the parasites. Although there sometimes appeared to be an increase in number of organisms after 8 to 18 hours incubation, it is probable that this appearance was due merely to the enlargement of the parasites. The occasional observation of isolated, small clusters of parasites probably indicates multiplication prior to rather than after cultivation, the products of "schizogony" being prevented from dispersion by excision of the tissue and cessation of blood flow.

To verify this, the pericardial fluid of a heavily infected oyster was cultured in thioglycollate medium maintained under a cover slip. The preparation was sealed with petrolatum to prevent evaporation

and movement of the fluid. An area containing a few parasites was focused upon and photomicrographs were made at varying intervals (30 minutes to 24 hours). From such series of photomicrographs the gradual increase in size of the parasites can be followed, but no increase in the number of organisms can be observed (Figs. 4 and 5). Two series of photomicrographs of cells containing a number of daughter cells, cultured in the manner described above, show that each of the daughter cells enlarges gradually, and the enlarged daughter cells remain in a compact group forming a cluster even in a liquid medium (Fig. 6). Based on the evidence obtained in the above studies it is considered unlikely that multiplication prior to enlargement of the parasites occurs to an extent that would invalidate the use of this technique to estimate the intensity of D. marinum infections in oysters.

A method for estimating the intensity of infection in cultured tissues was devised (Ray et al., 1953) in which three intensities of infection (light, moderate, and heavy) were defined. Later the writer (Ray, 1953a) further subdivided the three intensities into very light, light to moderate, and moderate to heavy. While a heavy infection is sharply separable from a light infection, the limits of the intervening categories are not easily defined, for they are largely a matter of personal judgment based on more or less extensive experience. The criteria used for determining the six categories of infection intensities are briefly described below:

1. A heavy infection is one in which the parasites occur in such enormous numbers that the major part of the tissue macroscopically shows a dull green-blue to blue-black color after treatment with iodine. With occasional exceptions, microscopic examination is not necessary for diagnosis of heavy infections. In a very few instances of heavy infection, macroscopic diagnosis is not possible because the organisms fail to enlarge or to stain normally after the usual treatment. However, in such cases microscopic examination reveals the heavy nature of the infection since the tissues are packed with the cells of the parasites.

2. A moderate to heavy infection is one in which the parasites are present in large numbers. This intensity differs from a heavy infection in that less than one-half of the tissue gives a macroscopic blue reaction with iodine. On the other hand, a moderate to heavy infection differs from a moderate infection in that some localized areas of macroscopic blue color appear after the iodine treatment.

3. A moderate infection is one in which the organisms are so numerous in the tissues that one may expect to find some in every field at 100X magnification. The masses of the cells of the parasites, however, are more or less localized, i.e., there are concentrations of parasites in some areas while others have relatively few of them. A moderate infection does not show a macroscopic blue color when the tissue is treated with iodine, although sometimes in cultures more than 72 hours old the tissues may be slightly tinged

with blue. This may also occur when the majority of the organisms enlarge to an unusual degree (100 to 150 μ in diameter).

4. A light to moderate infection is one in which some areas may be entirely free of the parasites and other areas may show localized concentrations containing something like 25 to 50 parasitic cells, or one in which the organisms may be scattered throughout the preparation more or less uniformly so that two or three parasites may be seen in each field at 100X magnification.

5. A light infection will show more than 10 organisms up to approximately 100 in the entire preparation. In such infections the parasites may be scattered singly in the tissues, or they may occur in isolated clusters of up to 10 to 15 organisms. The total number of organisms per preparation is not used alone to define the upper limits of a light infection; consideration is also given to the concentration of the parasites with regard to the amount of tissue being examined.

6. A very light infection consists of finding one to ten organisms in an entire tissue preparation.

The estimated intensities are used to derive the "weighted incidence" for each sample of oysters checked. The system used herein for determining the weighted incidence is that devised by Mackin (unpublished), in which a numerical value (weighted incidence) is obtained by arbitrarily assigning values of one-half, one, two, three, four, and five, to very light, light, light to moderate, moder-

ate, moderate to heavy, and heavy infections respectively. The sum of these values, when divided by the number of individuals in the sample, gives the weighted incidence per oyster. The weighted incidence, which combines incidence and intensity of infection, provides a much better index of the degree of infection than incidence alone. (See Figs. 25 and 26).

This method of diagnosis has these advantages over the histological techniques usually employed: (1) economy of time, materials, and labor, (2) diminution of equipment and skill required for a reliable diagnosis, and (3) increased accuracy in the detection of light infections because a greater amount of tissue is examined. Since dead and badly decomposed tissues usually make unsatisfactory histological preparation, this technique has an added advantage in that it permits diagnosis of D. marinum in dead or decomposing oysters. The cost of the medium and antibiotics for a single diagnosis is less than two cents.

The thioglycollate culture technique has been extensively used in the study of the distribution of D. marinum, and it has greatly facilitated experimental studies of the biology of this parasite.

As yet, the biological significance of the enlarged forms developing in culture remains obscure. Enlarged forms of Dermocystidium have been observed with extreme rarity in uncultured oyster tissues. Owen (unpublished data) found four oysters that contained large forms of Dermocystidium which possessed a distinct cyst wall,

the maximum size being 40 μ. These oysters were maintained at about 25° C. during the winter. The writer has found one live oyster with forms reaching 40 μ. This oyster was from a group that remained in transit (unrefrigerated) for four days in June. These organisms did not stain blue when treated with iodine; when cultured these forms enlarged further in the usual manner and stained blue when treated with iodine. The rare occurrence of somewhat enlarged forms in oysters indicates that the enlargement of the parasites is not a phenomenon restricted entirely to artificial cultural conditions. There is some evidence which suggests that these enlarged forms are abortive sporangial bodies. The cultured forms have not proven to be infective in vitro; in vivo infection attempts, however, have been inconclusive. Infected tissue mince cultured in thioglycollate medium for 72 hours, as well as masses containing enlarged bodies teased from tissues cultured for the same period of time, have produced infections when "injected" into uninfected oysters. Since a large number of normal size or only slightly enlarged organisms were observed in both preparations, it is not believed therefore that the enlarged forms caused the infections, but rather that the unenlarged forms were responsible.

2. Nutritional Studies.

Following the discovery that D. marinum would enlarge in fluid thioglycollate medium, studies were made to determine what constituents of this medium are necessary for enlargement. Oyster

tissues were incubated in media prepared by dissolving the individual ingredients of fluid thioglycollate medium (Difco) as well as the various combinations of them in sea water, to give similar concentrations per liter as follows:

1. Bacto-yeast extract - - - - - - - - - - - - - - - - - - 5 g.
   (water soluble portion of autolysed yeast)
2. Bacto-casitone - 15 g.
3. Dextrose - 5 g.
4. Sodium chloride - - - - - - - - - - - - - - - - - - - 2.5 g.
5. l-Cystine, Difco - - - - - - - - - - - - - - - - - - - 0.75 g.
6. Thioglycollic acid - - - - - - - - - - - - - - - - - - 0.3 ml.
7. Bacto-agar - 0.75 g.

In all cases where yeast extract and dextrose were used together, the growth of the parasites was nearly comparable to that occurring in the thioglycollate medium except that the walls appeared slightly thinner. Enlargement occurred frequently in mantle tissues when yeast extract alone was employed but never in gill tissues. This difference may be due to the fact that gill tissues contain considerably less glycogen than mantle tissues. Glycogen may provide the carbohydrates necessary for growth. It was also observed that some pieces of mantle tissue from the same oyster would show enlarged parasites in the absence of dextrose, while others would not; the pieces that did not show enlarged organisms were usually thinner or less opaque than the others. This thinness usually indicates a reduced glycogen content.

In a few cases some enlargement occurred with casitone and dextrose, but there were several failures when this combination was used. With the exception of yeast extract and casitone none

of the other ingredients, either singly or in combinations, were found to induce growth of the parasites within 14 days. Some enlarged forms were found in a very few cultures containing only dextrose after 36 days' incubation (cultures not examined between the 14th and 36th days).

In view of the fact that B-complex vitamins are important constituents of yeast extract, the possibility that vitamins may be the principal active constituents of yeast extract was investigated. Oyster tissues were incubated in media consisting of various vitamins and dextrose (5 gms./liter). The vitamins and the concentrations (mgs./ml.) used were as follows: thiamine HCl (0.02); riboflavine (0.007); nicotinic acid (0.06); calcium pantothenate (0.02); pyridoxine HCl (0.004); p-aminobenzoic acid (0.04); folic acid (0.003); choline chloride (0.40); inositol (0.50); ascorbic acid (0.001); biotin (0.0004); and B_{12} (0.0003). For the most part these concentrations corresponded to amounts found in analysis of yeast or yeast extract, or the amounts recommended for biological assay media. Cultures containing the individual vitamins and various combinations of them, including the entire aggregate, showed no enlarged parasites. These results indicate that some or all of the other components of yeast extract are necessary for rapid enlargement.

Mackin (unpublished data) found that considerable enlargement of D. marinum occurred occasionally in mantle tissues (from "gapers") incubated in sea water fortified with antibiotics, when large pieces

of mantle tissue (about 1 gram in 10 mls. of sea water) were used; however, the rate of growth as well as the number enlarging was much less than in thioglycollate medium. This finding is contrary to what the writer has observed when small pieces of gill and mantle tissue (5 x 10 mm.) from live oysters were incubated under similar conditions. It is not known how long and how extensively ciliary activity was maintained in the large pieces of mantle tissue used by Mackin, but it is suspected by the writer that these tissues died in a relatively short time, whereas small pieces of mantle tissue have been observed to maintain ciliary activity for as long as 30 to 40 days. It is possible that upon death the large pieces of tissue may produce by autolysis the required nutritive substances in sufficient quantities to induce enlargement of some of the parasites.

A series of observations were made which seem to support this suggestion. Small pieces of gill and mantle tissues (approximately 5 x 10 mm.) from a heavily infected "gaper" were planted in tubes containing 10 mls. of sterile sea water fortified with antibiotics. At the time of planting, some pieces of the mantle and all of the gill tissues showed ciliary activity. Pieces of these tissues stained with iodine showed neither blue colored organisms nor organisms that could be observed at 100X magnification. The results obtained are tabulated below:

Tissue	Period of incubation	Ciliary activity	Visible at 100X	Blue color
Gill	24 hours	strong	none	none
Mantle	24 hours	none	many	none
Gill	48 hours	strong	none	none
Mantle	48 hours	none	many[1]	light blue & green[1]
Gill	72 hours	strong	none	none
Mantle	72 hours	none	many[1]	light blue & green[1]
Gill	96 hours	strong	none	none
Mantle	96 hours	some	none	none
Gill	5 days	strong	none	none
Mantle	5 days	some	none	none
Gill	8 days	strong	none	none
Mantle	8 days	none	very many	blue & green (5 to 77 μ)
Gill	11 days	strong	none	none
Mantle	11 days	none	very many	strong (max. 75 μ, majority 15 to 40 μ)
Gill	16 days	none	none	none
Mantle	16 days	none	none	none

[1] Appearance similar to 18 hour thioglycollate cultures.

The above results suggest that death of the tissue is necessary for enlargement of D. marinum in sea water alone, although enlargement will occur in living tissues if sufficient nutrients are available.

When D. marinum is cultured in medium containing yeast extract and dextrose, some individuals germinate to produce a single, short hypha or germ tube which possesses a terminal, budlike process (Fig. 7); on rare occasions two hyphae have been observed (Fig. 8). It was previously reported (Ray, 1952a) that hyphal forms were produced consistently when the parasites were cultured in yeast extract and dextrose; further work has shown that this is not true. Since these forms have been observed more frequently in the colder months than in the warmer ones, it was considered that the

generally lower room temperatures during the colder months may stimulate the germination of the cultured parasites. For example, Blastomyces dermatitidis produces a yeastlike organism (The tissue form) when cultured at 37° C., whereas a mycelial form develops at room temperature. Incubation of cultures during the summer months at both 18° and 30° C. produced only rare hyphal forms.

As previously mentioned, the hyphal forms have been found more frequently in thioglycollate medium rehydrated with distilled water than with sea water or distilled water containing 20 grams NaCl per liter. This suggested that salinity may be a controlling factor for the production of such forms. The salinity of the yeast extract and dextrose medium was varied from 2.5/1000 to 25/1000 and the cultures were incubated at 18° and 30° C. Under these conditions during the summer, hyphal forms were observed only infrequently and there was no evidence that any particular set of conditions increased the production of these forms. Similar results were obtained during the fall and winter, except that hyphal forms were found more frequently. The above evidence suggests that the condition of the parasites or the host tissues is more important in influencing the germination of the cultured parasites than any of the experimental factors thus far considered. These forms appear to occur more frequently in a medium containing yeast extract and dextrose than in other media (fluid thioglycollate, mycophil broth "BBL", and nutrient broth).

Since many of the conventional media for fungi contain relatively high concentrations of sugar (as high as 40 gms./liter), the possibility that increased sugar concentrations may stimulate germination was considered. Tissues were planted in media containing yeast extract (5 gms./liter) and dextrose in concentrations of 5, 10, 20 and 40 grams per liter. These cultures were incubated at 18° to 20°, 22° to 25°, 30°, and 37° C. and were examined after 15 days incubation. At 37° C. the parasites died. There was a marked retardation of growth and of the blue reaction of the parasites in the 40-gram cultures; the maximum size observed was 18 μ as compared to 60 μ in 5, 10, and 20-gram cultures. The average size of the parasites appeared to be somewhat less in the 20-gram series. Some hyphal forms were present in all but the 40-gram series, but there was no evidence that any of the three sugar concentrations in combination with the three ranges of incubation temperatures stimulated germination.

3. Miscellaneous Studies.

Cultured parasites inoculated on various solid media showed no evidence of growth or reproduction. The following media rehydrated with sea water were used: yeast dextrose agar, mycophil agar, Littman's oxgall agar, Sabouraud dextrose agar, and sea water agar. Negative results were also obtained with the above media containing 10 per cent oyster tissue mince.

The salinity and temperature tolerances of D. marinum in thioglycollate medium were investigated. The temperature studies will be discussed first. The optimum temperature for enlargement appears to be 30° C. (Langhorne, unpublished data); but the rate of growth at 25° C. is only slightly less. No enlargement was observed after incubation at 0° to 4° C. for 48 hours; cultures that were maintained at this range of temperature for about 60 hours and then returned to room temperature showed only slightly less growth than the control cultures. After 10 days, however, at 0° to 4° C. many parasites died and only a small amount of enlargement occurred after subsequent incubation at room temperature. Considerable growth of the parasites occurred at 10° C. after 48 hours, but the average size was less than in the control cultures incubated at room temperature (17° to 24° C). Growth is considerably retarded at 35° C. after 48 hours. When cultures incubated at this temperature for 60 hours were returned to room temperature, no appreciable growth took place. The great majority of the parasites died after 48 to 60 hours incubation at 35° to 37° C. From the preceding results it is evident that growth of D. marinum in culture occurs over a considerable range of temperature (10° to 30° C).

The effects of salinity, varied over a wide range, on the growth of D. marinum in thioglycollate medium were extensively studied. At salinities of 1.5/1000 or less, the great majority of the organisms were destroyed and only a few swollen, thin-walled organisms which

stained a faint blue were observed. Between salinities of 1.5 and 3.5/1000 a few extremely large, thin-walled parasites were noticed, but the majority of them were fragmented. Thioglycollate medium rehydrated with distilled water has a salinity of approximately 4/1000 after sterilization. With occasional exceptions, no appreciable difference has been observed in the enlargement of the parasites in medium rehydrated with distilled water or sea water. The walls of the parasites, however, usually become thicker when sea water is used; consequently, the parasites give a more intense blue reaction with iodine.

The parasites showed comparable growth in medium adjusted to the same salinities regardless of whether sea water or distilled water containing NaCl was used for rehydration. The growth appeared to be more uniform with distilled water containing NaCl. The above observation led to the recommendation (Ray, 1953) that distilled water containing 20 grams NaCl per liter be used to rehydrate thioglycollate medium. Since estuarine waters vary considerably from time to time with regard to salt content as well as other dissolved substances that may possibly influence the growth of the parasites, this modification should add to the uniformity of the culture method of diagnosis, especially with regard to the estimation of intensity of infection. Thioglycollate medium rehydrated in the manner suggested above has a salinity of approximately 25/1000 after sterilization. The enlargement of parasites cultured in medium with a

salinity of about 50/1000 was only slightly less than the enlargement of those cultured at approximately 25/1000. The above results show that in culture D. marinum has an extremely wide range of salinity tolerance.

On one occasion during this investigation it was observed that D. marinum from a group of oysters maintained in closed aquaria showed practically no enlargement or blue reaction after cultivation. With regard to conditions of cultivation, no factors such as temperature, salinity, or bacterial growth were found to account for the failure of the parasites to grow. In these aquaria distilled water (prepared in copper stills) was used over a period of several weeks to replace water evaporating from them, instead of using tap water (allowed to stand for a week) as had been the previous practice. It was considered that copper, a well-known fungicide, may possibly have accumulated in sufficient concentrations to inhibit or kill the parasites. Unfortunately time was not available to permit copper determinations of the distilled water and the water in the aquaria to possibly verify this suspicion. With the return to the practice of using tap water to replace evaporating water, failures in growth (such as mentioned above) have not been encountered subsequently.

In order to obtain some information on the effects of copper on D. marinum, small pieces of infected oyster tissue were placed in sea water fortified with antibiotics and containing various concentra-

tions of copper sulfate ($CuSO_4 \cdot 5H_2O$). The tissues were incubated in the sea water cultures at room temperature for about 66 hours and about 8 days, then placed in thioglycollate medium after being washed in sterile sea water to remove as much copper sulfate as possible.

The parasites subjected to concentrations of copper sulfate ranging from about 7.5 ppm. to about 150 ppm. for 66 hours showed growth comparable to that occurring in the control cultures. The average size of the organisms in the 7.5 ppm. and 15 ppm. cultures was greater than that of the controls. No enlargement or blue reaction was observed at a concentration of 750 ppm. after 66 hours. After eight days incubation the growth was noticeably greater in the cultures containing 7.5 ppm. copper sulfate than in control cultures and slightly greater in the 15 ppm. cultures. The number of organisms enlarging in the 75 ppm. and 150 ppm. cultures was greatly reduced, although a few large ones were observed. Just as in the 66 hour series, no enlargement or blue reaction was obtained at copper sulfate concentrations of 750 ppm.

The above studies give no indication of the actual concentrations of copper ions to which the organisms were subjected, but they do demonstrate that under cultural conditions small amounts of copper sulfate stimulate growth of D. marinum and moderate amounts of this compound kill the parasites.

Figure 1. *D. marinum* in oyster gill tissue after 35 days of incubation in fluid thioglycollate medium; x 200.

FIGURE 1.

Figure 2. D. marinum in oyster heart tissue after 23 days of incubation in fluid thioglycollate medium, iodine stained; x 240.

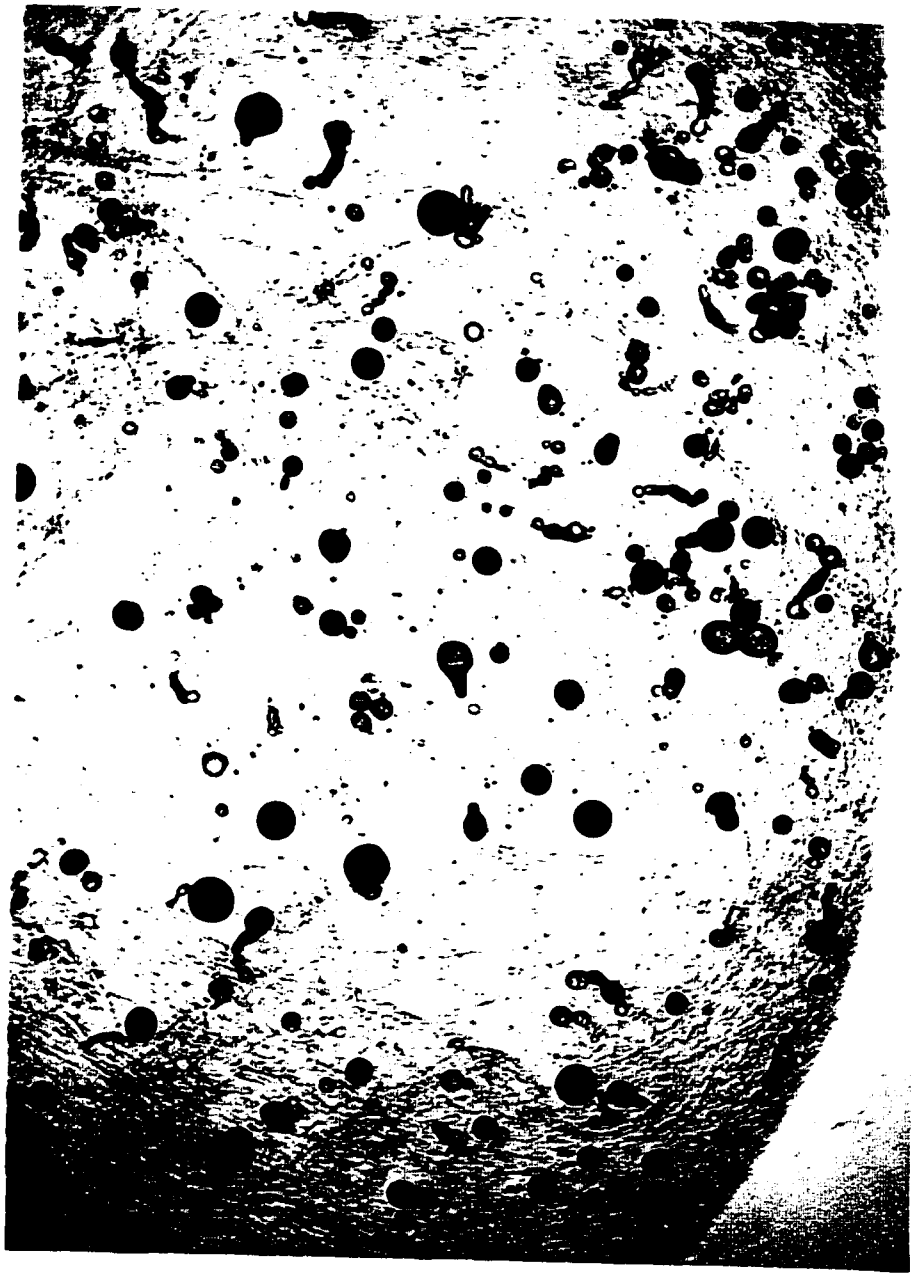
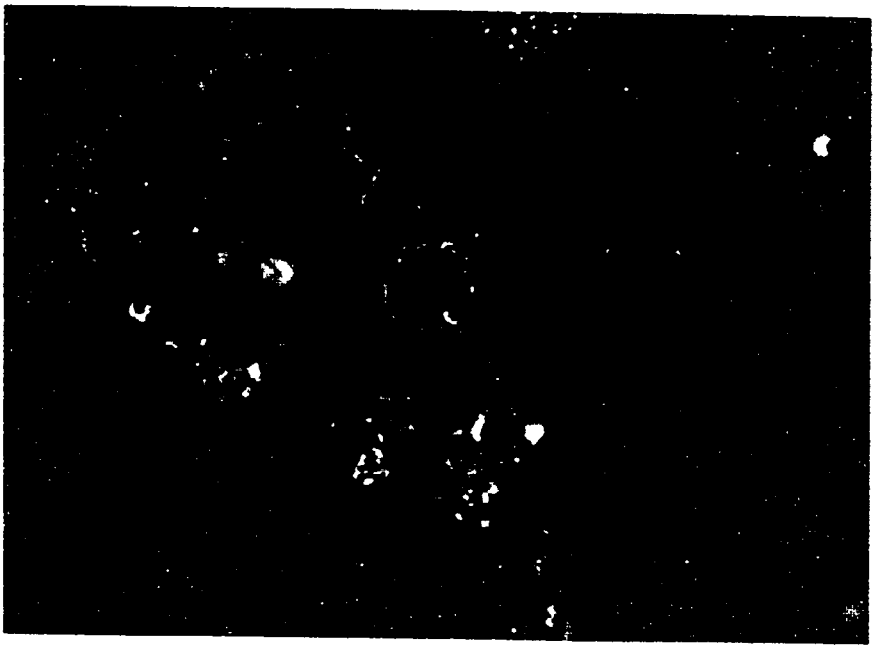


FIGURE 2.

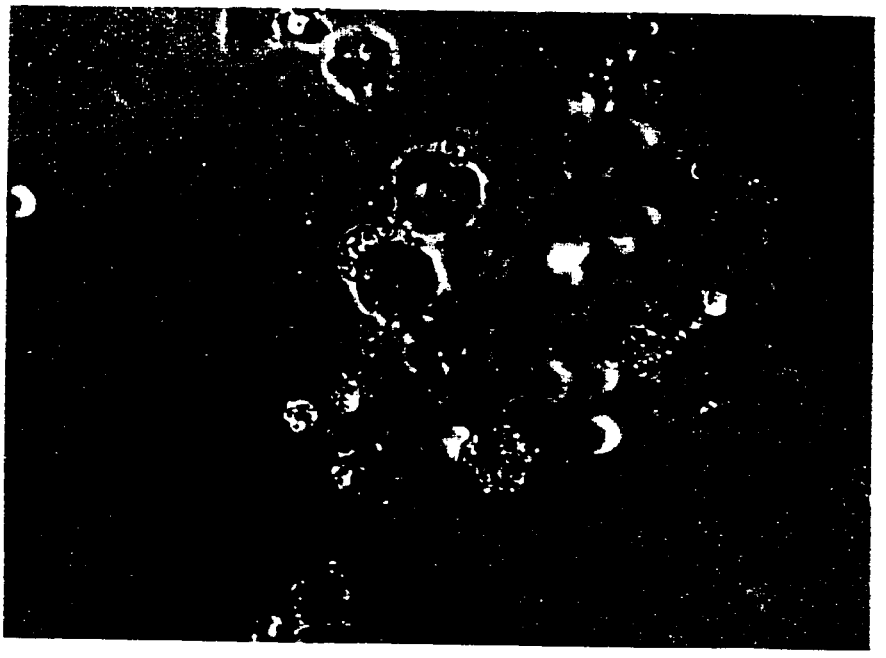
Figure 3. *D. marinum* in oyster heart tissue after 23 days of incubation in fluid thioglycollate medium, iodine stained; x 260.

FIGURE 3.

Figure 4. Photomicrographs of oyster pericardial fluid cultured in fluid thioglycollate medium, showing D. marinum, oyster leucocytes, and cellular debris. A: initial exposure made shortly after culturing. The gradual enlargement of the four marked parasites can be easily followed. Parasite 1 is within a leucocyte. Note: A was printed in reverse of the other figures in this series. B: after 2 hours in culture. All figures x 550. See Fig. 5 for remainder of the series.



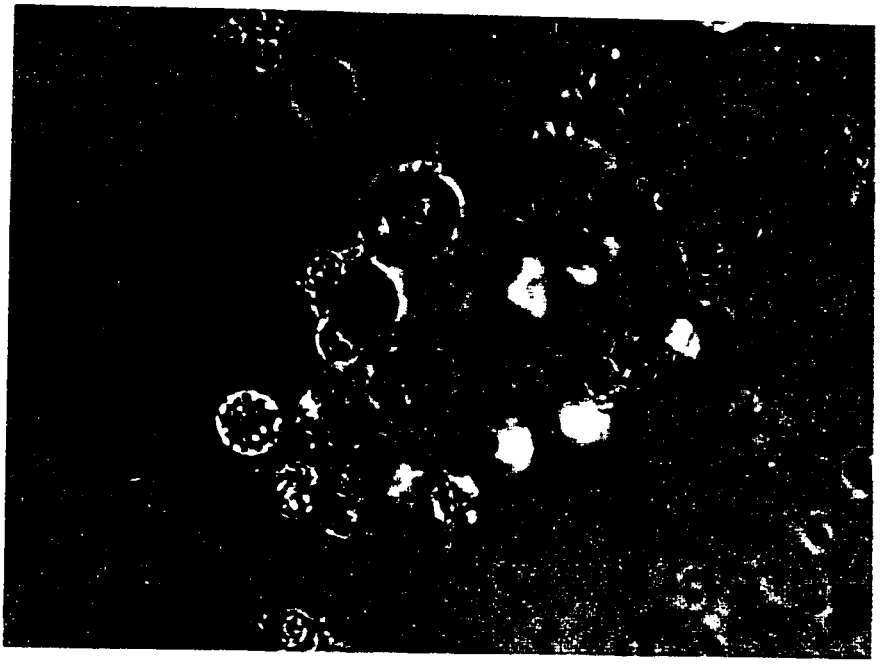
A



B

FIGURE 4.

Figure 5. Later exposures of the culture presented in Fig. 4. A: after 6-1/2 hours in culture. Note the displacement of the cytoplasmic contents by enlargement of parasite 1. B: after 19 hours in culture. Note rupture of leucocyte cell wall by enlargement of parasite 1. All figures x 550.

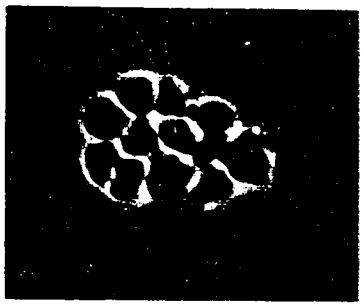


A

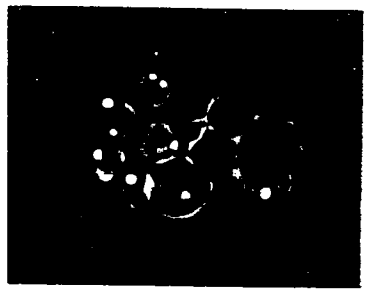
B

FIGURE 5.

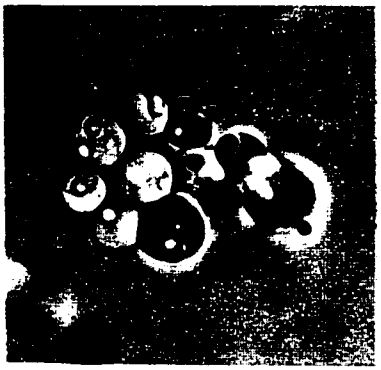
Figure 6. Photomicrographs of the progressive enlargement of the daughter cells of a reproducing form of D. marinum from oyster pericardial fluid cultured in fluid thioglycollate medium. Note: the 12 daughter cells are in clear focus only in B and E. A, after 20 minutes; B, after 2-1/3 hours; C, after 4-1/3 hours; D, after 6-1/2 hours; and E, after 21 hours. All figures x 1000.



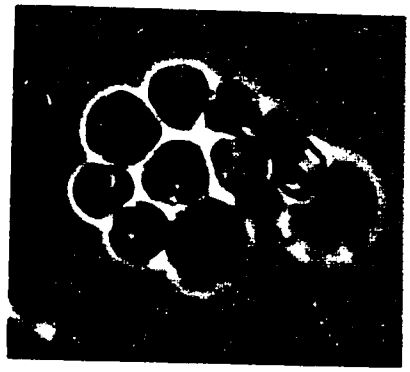
A



B



C



D

E

FIGURE 6.

Figure 7. D. marinum from oyster mantle tissue after 41 days incubation in sea water containing yeast extract and dextrose, iodine stained; x 260.

FIGURE 7.

Figure 8. D. marinum from oyster mantle tissue, one organism with two hyphae, incubated for 41 days in sea water containing yeast extract and dextrose, iodine stained; x 260. Note: body in upper left hand corner with non-staining periphery is a contaminant.

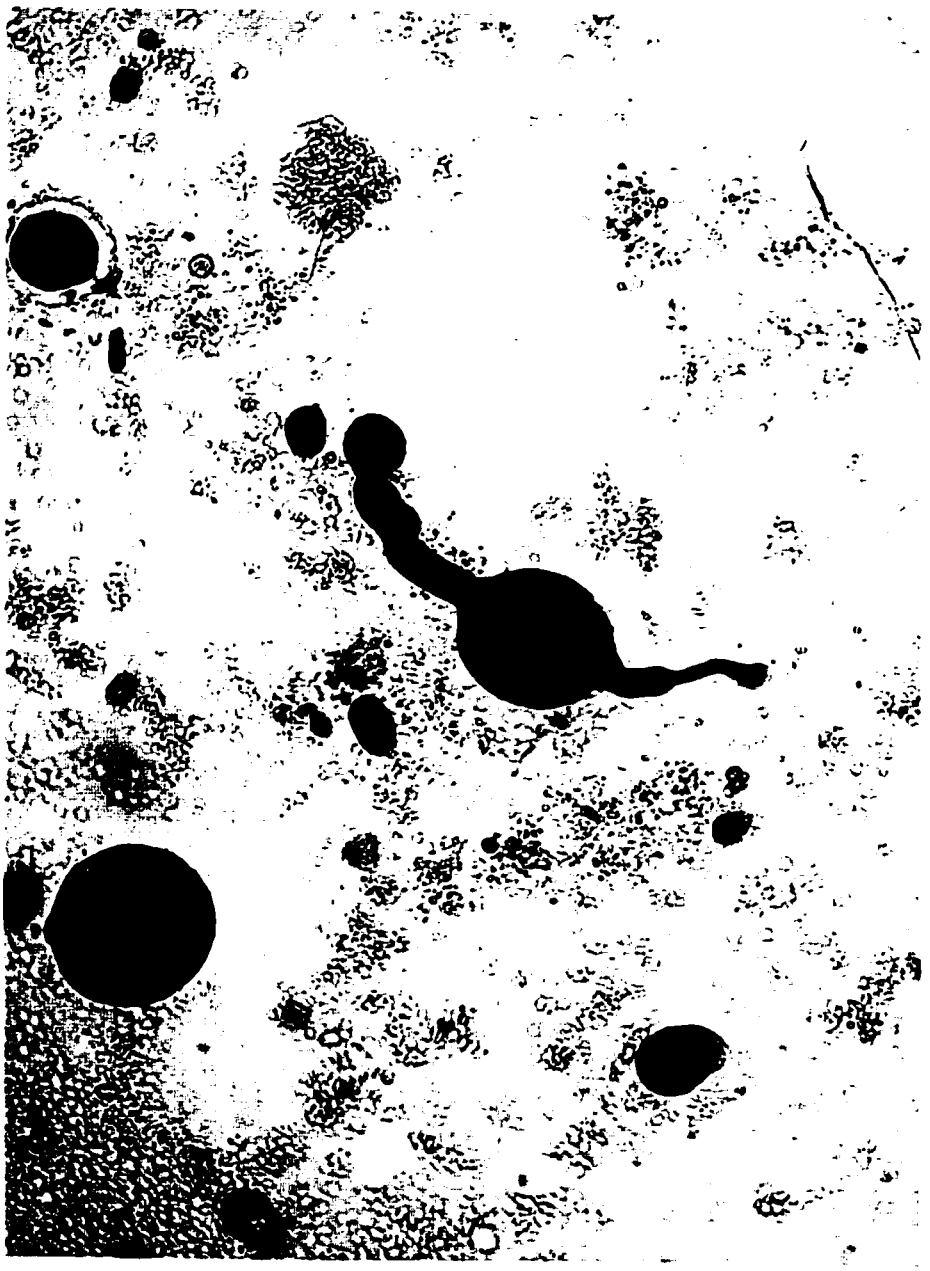


FIGURE 8.

TRANSMISSION AND PATHOGENICITY STUDIES

1. Transmission Under Natural Conditions.

Studies were conducted to determine the time required for oysters from non-endemic areas to become infected when placed in water in which D. marinum is endemic. In one study a group of oysters estimated to be four years old was obtained from Gardiner's Bay, Long Island, New York, on August 21, 1952. These oysters were maintained in trays in Bayou Rigaud, Grand Isle, Louisiana. This is a locality with heavy natural Dermocystidium infections where, as will be explained later, only young oysters thrive, except in winter. The trays were located just a few feet from trays containing infected oysters. A sample of 30 oysters from this group was examined on August 22, 1952, and all were found to be free of the parasite. Ten oysters checked after being in endemic waters for one week were negative for D. marinum. After three weeks a sample of similar size proved to be 20 per cent infected (weighted incidence of 0.15). In this group of oysters the time required for the initial appearance of the parasite was between one and three weeks. Four weeks after these oysters were placed in Bayou Rigaud, they were found to be 30 per cent infected (weighted incidence of 0.25). After the same period of time another group of Long Island oysters, which had been maintained in a tray containing a large number of infected oysters (incidence of 65 per cent; weighted incidence of 1.18), was fou nd to have an incidence of 60 per cent (weighted incidence of 0.95).

In both groups of oysters the first heavily infected "gapers" were recovered approximately six weeks after the initiation of the studies. Except for deaths occurring immediately after shipment, the mortality remained relatively low until the following spring (see Table 8). During the fall there occurred some deaths which were probably due to D. marinum; several heavily infected "gapers" were recovered during October and November. The incidence of infection averaged about 60 per cent and the weighted incidence ranged from 1.2 to 2.0 during the month of October. Since the weighted incidence had reached a fairly high level, the writer believes that mortality would have increased considerably within a short time had it not been for the reduction of the water temperatures during the latter part of October. The incidence and intensity of D. marinum in this group of oysters was observed for nearly a year; the results will be discussed later (Part VI, dealing with the incidence of D. marinum in oysters of various ages).

On June 12, 1953, a large number of uninfected oysters (about three years old) from Milford, Connecticut, were placed in Bayou Rigaud in a tray which was adjacent to trays containing infected oysters. Beginning one week later, a sample of ten oysters was checked at weekly intervals for four consecutive weeks; the incidence and weighted incidence for the first to the fourth week respectively was as follows: 1 oyster (10 per cent, 0.05); 8 oysters (80 per cent, 0.85); all 10 oysters (100 per cent, 1.70); and 9 oysters (90 per cent, 2.25).

The first heavily infected "gaper" was recovered about 18 days after the start of the study and the mortality continued fairly steadily throughout the summer and fall until the last week in October. At this time the mortality dropped to zero. On July 1, 1953, when the first heavily infected "gaper" was found, there were 77 live oysters on hand, and by October 27, 1953, 61 (80 per cent) of the oysters had died. Fifty "gapers" (82 per cent of those oysters dying) were recovered and all but one was found to be heavily infected with D. marinum. The average water temperatures ranged between 28° and 31° C. during September. Of the 16 oysters surviving after October 27, 1953, three died (two in December and one in February) by March 18, 1954. Two of these oysters were heavily infected and the third was negative. The water temperatures averaged less than 25° C. during the first part of October and about 20° C. during the latter part.

The results of the studies presented above show that oysters from non-endemic waters begin to pick up D. marinum between one and three weeks. The data also indicate that the incidence and intensity of infection increase with surprising rapidity, and after three to six weeks the infections reach intensities sufficient to cause death during the warmer months. If one excludes the possibility that each of the groups of oysters used was not equally susceptible to infection, the data suggest, as one would expect, that uninfected oysters placed in endemic waters during early June will sustain a much higher mortality during the first summer than those placed in such waters during

the latter part of August. The importance of this fact with regard to oyster cultivation will be discussed later.

In the preceding studies the observation that the rate of infection was increased by proximity to infected oysters suggests that Dermocystidium disease is contagious. Studies by Mackin (1951b) indicate the contagious nature of this disease. He found that oysters having a low incidence of D. marinum and receiving water from an endemic source (Bayou Rigaud) after it had passed through an aquarium containing oysters with a high incidence of the parasite, showed a greater incidence and a greater mortality than a similar group of oysters receiving water from the same source that had passed through an aquarium containing oysters with a relatively low incidence of the parasites. These observations suggest that D. marinum may be transmitted directly.

2. Experimental Transmission.

The following studies were conducted to determine how D. marinum may be transmitted from oyster to oyster. This information is necessary for laboratory investigation of the pathogenicity of the parasite under controlled conditions. The initial phases (Ray, 1954) of the studies, which include experiments R3-1 and R3-2, were conducted at Rice Institute. The other studies (presented as two papers at the annual meeting of the Texas Academy of Science, Galveston, Texas, December, 1953, by Ray and Mackin, and by Mackin,

Ray, and Boswell) dealing with experimental transmission were conducted in collaboration with J. G. Mackin and J. L. Boswell, the work being done at the Texas A. & M. Research Foundation Laboratory at Grand Isle, Louisiana. Four methods of transmission were investigated, namely: "proximity", "feeding", "injection", and in vitro. The procedures followed and the results obtained with each of the methods are presented below.

a. "Proximity" Method.

1. Experiment R3-1.

Experiments R3-1 and R3-2 were carried out simultaneously, using closed aquaria. Each aquarium contained approximately 10 liters of sea water from Galveston Bay, Texas. The water in each aquarium was aerated continuously and was renewed weekly or, in a few instances, at 10 to 14 day intervals. Approximately two liters of water were left in each aquarium and eight liters of fresh sea water added at each change. Since some evaporation took place, the salinity and pH were determined before and after the water was changed. The salinity values ranged from 19 to 34/1000 (See Fig. 10). The water temperatures were recorded at least once a day. The pH of the water varied from 7.1 to 8.1 during the course of the experiments; for the great majority of the time, however, it ranged from 7.5 to 7.8. All aquaria were checked for "gapers" at least once a day.

The uninfected oysters used in these experiments were obtained from Milford, Connecticut, and were approximately two and one-half

years old. A sample of 28 oysters from the same group checked for D. marinum by J. R. Uzmann at Milford, Connecticut, before shipping proved to be negative for the parasites. The Milford oysters were received in Houston, Texas, on December 9, 1952; they appeared to be in very good condition in spite of the fact that they had been out of water for nine days. The infected oysters used were obtained from storage trays at Grand Isle, Louisiana, and Galveston Bay, Texas.

In experiment R3-1, which is designated as a "proximity" study, four experimental aquaria and one control aquarium were set up on December 9 and 10. Each experimental aquarium contained ten presumably uninfected Milford oysters and five possibly infected Louisiana oysters. The control aquarium contained 15 Milford oysters. Two of the aquaria contained Louisiana oysters that were received on December 3 and two contained oysters that were received from the same source on December 10. From the beginning of the study until April 2, 1953, each oyster from an endemic area that gaped was replaced with an oyster from Louisiana or Texas. The data on the Louisiana and Texas oysters used in this study are presented in Fig. 9 and Table 1.

A number of tissues (gill, mantle, heart, and rectum) of all "gapers" and survivors were checked for D. marinum and the intensity of infection was estimated. This study was discontinued on May 2, 1953, when 50 per cent of the Milford oysters in the experimental aquaria had died.

The results of this study are presented in Fig. 9 and Table 3.

In Fig. 9 each dead oyster is represented by a block which indicates by its shading the intensity of infection. The period covered by this study is divided into weekly intervals, and each block is plotted to indicate the week in which the death occurred. Throughout the studies on transmission and pathogenicity the intensities recorded are based on the estimates obtained by examination of rectal and mantle tissues which have been found to be the best indicators.

The oysters from the endemic areas were found to be 100 per cent infected with the parasite. Forty-four (86 per cent) of 51 Louisiana and Texas oysters died during the course of this experiment, which lasted nearly five months. Forty-three (98 per cent) of the "gapers" had infections ranging from moderate to heavy. Since it has been shown (Ray, Mackin, and Boswell, 1953) that such intense infections with D. marinum produce a definite diseased condition manifested by an appreciable loss of weight, these oysters are considered to have been in an acute stage of Dermocystidium disease, which is believed to have been an important contributing factor to their deaths. Throughout this thesis oysters with moderate or greater intensities will be referred to as acutely infected or acutely diseased.

This study was discontinued when 20 (50 per cent) of 40 Milford oysters in the experimental aquaria had died. Thirty-eight (95 per cent) of the Milford oysters had become infected, and 12 (60 per cent of the dead oysters showed acute infections. Three (20 per cent) of the 15 uninfected control oysters died during the experiment. It

may therefore be presumed that 20 per cent (8) of the Milford oysters in the experimental aquaria died of causes other than Dermocystidium infection, as did those in the control aquarium. Fig. 9 shows that eight Milford oysters in the experimental aquaria had less than acute infections with D. marinum at time of death. All of the control oysters including the "gapers" and survivors were negative for the parasite. Since 13 (65 per cent) of the surviving Milford oysters in the experimental aquaria had heavy intensities of infection, it is likely that a large portion of the survivors would soon have died of Dermocystidium disease.

The results of the "proximity" study demonstrate that D. marinum may be transmitted from oyster to oyster. Furthermore, they show that the mortality of the experimental oysters was 2.5 times greater than that of the control oysters.

b. "Feeding" Method.

1. Experiment R3-2.

This study, which is designated as a "feeding" experiment, consisted of placing finely minced tissues of oysters with varying degrees of infection in the experimental aquaria and finely minced tissues of uninfected oysters in the control aquaria. The tissue minces were prepared by cutting the oysters into small pieces, then finely mincing in a Waring Blendor with approximately 25 mls. of sterile sea water per oyster. The Blendor was operated intermittently for several periods of a few seconds each. This procedure keeps the speed of the

cutting blades reduced so that the majority of the parasites will not be damaged when infected oysters are being minced. Two experimental and one control aquaria, each containing 15 uninfected Milford oysters, were used. Cultures were made to be sure that viable organisms were present in the infected material and absent in the control material. The experimental oysters received five "feedings" of infected tissues at irregular intervals whereas the control oysters received only one "feeding" of tissue mince prepared from one of the Milford oysters. See Table 2 for dates of "feeding" and other data regarding oysters used to prepare the minces. The experiment was discontinued May 1, 1953.

The results of this study are presented in Fig. 10 and Table 3. The mean weekly temperature and mean weekly salinity for experiments R3-1 and R3-2 are plotted in Fig. 10. Six (40 per cent) of the 15 control oysters died during the experiment. All of the control oysters were found to be negative for D. marinum. Twenty-five (83 per cent) of the 30 experimental oysters died and 26 (87 per cent) of the experimental oysters became infected. Fifteen (60 per cent) of the oysters dying in the experimental aquaria showed acute infections with the parasite. Since 40 per cent of the control oysters died, it may be presumed that 40 per cent (12) of the experimental oysters died of the same causes as those in the control aquarium. An examination of Fig. 10 reveals that 10 of the experimental oysters were found to be less than acutely infected at the time of death.

The above results show that D. marinum may be effectively transmitted experimentally by the "feeding" method. The mortality of the oysters exposed to infected tissue mince was slightly more than twice as great as the mortality among the controls. However, since the experimental oysters received five "feedings" whereas the control oysters received only one, the above study is open to question with regard to the comparison of mortalities occurring in the control and experimental aquaria. There is the possibility that the greater amount of tissue decomposing in the experimental aquaria may have contributed to the increased mortality occurring in these aquaria. The following experiment, however, indicates that the "feeding" of tissue minces does not influence the mortality rate.

2. Experiment 36.

This experiment was a duplicate of the "feeding" study just described in which the control and experimental aquaria each received five "feedings" of five mls. each of appropriate tissue mince at weekly intervals. The oysters used in this experiment were obtained on July 13, 1953, from Redfish Bay, Louisiana, a low salinity area located near the mouth of the Mississippi River where thus far no infections have been found; the experiment was started on July 16. Each aquarium that was set up in this experiment contained 15 oysters. The experiment was run in duplicate; two experimental aquaria received heavily infected tissue mince and two control aquaria received uninfected tissue mince. Two additional control aquaria received no tissue

mince at all; they were set up in order to check the effect of decomposing oyster tissue in closed aquaria with regard to mortality of oysters. Each aquarium contained about 15 liters of sea water obtained along the beach at Grand Isle, Louisiana. The water was aerated continuously and approximately 12 liters were removed weekly and replaced with an equal amount of fresh sea water. Each aquarium was covered with a pane of glass to prevent excessive evaporation and to protect against the accidental introduction of infected material. This experiment was discontinued on September 11, after nearly two months duration. The salinity ranged from 24 to 28/1000 and the temperature varied through a range of 25° to 31° C. during the course of the experiment.

The results, which were strikingly similar in these duplicate experiments, are presented in Figs. 11 and 12, and Table 3. Since the results were so much alike, the data will be combined for the purpose of discussion. The mortalities in the aquaria receiving uninfected minces were 10 per cent (3 out of 30); the mortalities in the aquaria receiving no tissue were likewise 3 out of 30. These results indicate that decomposition of the tissue mince in the aquaria did not influence the mortality rates in this study. A mortality of 83 per cent (25 out of 30) occurred in the infected aquaria. This mortality was more than eight times greater than that occurring in both series of controls. All oysters, "gapers" and survivors, in the control aquaria were found to be free of D. marinum. On the contrary, all of

the experimental oysters examined were found to be infected except one that died one week after the initiation of the experiment. The death of an experimental oyster, indicated as a "box" in Fig. 12. was not detected until it had completely decomposed.

3. Experiment 28.

The following "feeding" experiment was conducted under more natural conditions than the preceding ones in that the oysters received circulating, filtered water from an infected locality (Bayou Rigaud) which was aerated continuously. Oysters, particularly those heavily attacked by boring sponge, survive better in circulating water than in non-circulating water (closed aquarium), especially during the warmer months. The use of circulating water reduces the possibility of aquaria becoming fouled by the accumulation of metabolic wastes and products of decomposition. The sea water was filtered by allowing it to pass by gravity flow through three glass wool filters arranged in tandem. It was necessary to change the glass wool in the filters at weekly intervals because they became clogged with silt. The flow of water from the last filter was divided so that each control and each experimental aquarium received approximately 300 liters per day which amounted to about 20 changes. The filters are shown in Fig. 15.

The oysters used in this experiment were obtained from Milford, Connecticut, on June 9, 1953, by air express. There was practically no mortality among these oysters when first received. However, the initial mortality was rather high in all experiments in which these

oysters were used. This mortality was assumed to be due to damage during transportation. The experiment was begun on June 11.

Twenty-five oysters were placed in the experimental aquarium and the same number in the control aquarium. Initially each aquarium received 20 mls. of appropriate mince and a week later 10 mls. were added to each one. The water temperatures ranged from 28° to 31° C. during this study.

The results are presented in Fig. 13 and Table 3. The overall mortality in the control aquarium was 52 per cent (13 out of 25) as compared to 96 per cent (24 out of 25) in the experimental aquarium. If, however, the initial mortalities occurring within a week and presumably resulting from transportation damage are excluded, the control oysters showed 37 per cent mortality while experimentals showed 94 per cent---approximately 2.5 times greater than that occurring in the control aquarium. All of the control oysters were negative for the parasite. The experimental oysters that died subsequent to the initial mortalities and the lone survivor were heavily infected with D. marinum.

4. Experiment 44.

This experiment was nearly an exact duplicate of the preceding one except that southern oysters obtained from Redfish Bay, Louisiana, were used; three "feedings" of 20 mls. each at weekly intervals were given instead of two. The experiment was begun on July 16, 1953, and discontinued on September 18. Water temperatures ranged from 25° to 31° C., being 28° C. or greater most of the time.

The results obtained in this experiment are presented in Fig. 14 and Table 3. The over-all mortality in the experimental aquarium was 92 per cent (23 out of 25) compared to 24 per cent (6 out of 25) in the control aquarium. If the initial mortalities occurring within three days (considered to have been due to culling damage since the oysters were thin-shelled) are excluded in computing the mortality percentages, the controls showed 14 per cent mortality while experimentals showed 91 per cent---6.5 times greater than that occurring in the control aquarium. All of the "gapers" and surviving control oysters were negative for D. marinum, whereas all experimental oysters that died subsequent to the initial mortalities were heavily infected with the parasite.

5. Additional "Feeding" Experiments.

In view of the fact that Nereid worms have occasionally been found infected with parasites appearing to be D. marinum, it was considered that an intermediate or transport host may be necessary for transmission of the parasite. To check this possibility, a "feeding" experiment was conducted in which the uninfected oysters were placed in a 0.1 per cent phenol solution for approximately two hours to kill the organisms in or on the shells. Several annelids were removed from the shells of the oysters by this treatment. Then the phenol was removed by rinsing in tap water. These oysters were maintained in a closed aquarium containing aerated, boiled sea water, and they received two "feedings" of heavily infected mince. The first heavily infected "gaper" appeared about one month after the initiation of the ex-

periment. The time required to obtain the first heavily infected "gaper" among untreated oysters maintained in unboiled sea water was about the same. This experiment does not preclude the existence of an intermediate or transport host, but it does indicate that such hosts are not necessary for transmission, at least under experimental conditions.

There is also the possibility that the infective stage of D. marinum may develop saprophytically after the decomposition of the host tissues containing the parasites. In order to investigate this possibility the following experiment was conducted. Uninfected oysters maintained in a closed aquarium received a single "feeding" of heavily infected mince (12 mls.) for six hours; then the oysters and aquarium were carefully scrubbed to remove all infected material other than that which the oyster retained within the shell. Assuming that the aquarium and the exterior of the oysters were completely freed of D. marinum, the only source of infective material would come from within the shell of the oyster. This fact, however, does not eliminate the possibility that some infected material may have been ejected later and then the saprophytic development of the infective stage could have taken place. Nevertheless, in view of the short time (24 to 48 hours) in which invasion and proliferation of D. marinum occurred in the in vitro studies to be discussed below, it is believed by the writer that the material initially taken in was infective. The first acutely infected "gaper" appeared 20 days after this experiment was begun.

c. In Vitro Method.

The "feeding" and "proximity" studies suggest that D. marinum may be transmitted directly; some further experiments which support this idea were performed. In vitro studies in which living, excised pieces of gill, mantle, and palp tissues from uninfected oysters maintained in sterile sea water fortified with streptomycin and penicillin were used to demonstrate the capacity of this parasite to invade other than intestinal epithelial tissues. Briefly, the procedure followed consisted of inoculating the tube containing the excised tissue with a drop of pericardial fluid obtained from a heavily infected "gaper" and subsequently culturing the tissues in thioglycollate medium after incubation at room temperature for periods of 24 hours or more. Examination of the cultured tissues showed no concentration of the parasites at the cut edges, although the majority showed a few to many isolated clusters of D. marinum throughout the tissues. These observations suggest the possibility that the organisms may penetrate the epithelium of the excised tissues and then proliferate rather rapidly.

Furthermore, after about two weeks' incubation, some of the tissues showed moderate and some moderate to heavy infections; these tissues, though maintaining some ciliary activity, showed signs of disintegration. They gave the appearance of undergoing histolysis since most of the clusters of organisms were surrounded by clear areas. During these studies some evidence which suggests that D. marinum will not invade and proliferate in dead tissue was obtained.

This evidence consisted of the observation that tissues showing some ciliary activity at the time of culturing in thioglycollate usually showed considerable multiplication of the invading organisms, whereas invasion and multiplication of the parasites was usually absent or, at most, very limited if the tissues failed to show ciliary activity at the time of culturing.

d. "Injection" Method.

1. Experiment 30.

This method of transmission was developed to insure the simultaneous infection of all experimental oysters and to accelerate the development of infections. Approximately 1 ml. of tissue mince (infected in case of experimental oysters and uninfected in case of the controls), diluted with sterile sea water to give about one-half the concentration used in the "feeding" studies, was introduced into the mantle cavity through a hole in the shell. Two holes usually 1/16 inch in diameter were drilled through the right valve of each oyster about 1/2 inch from the ventral shell margin (Fig. 16). Two holes were necessary to allow for the displacement of the shell liquor by the injected mince. A hypodermic syringe bearing a 15 gauge needle, which was rounded off at the end so that it would enter but not pass through the hole, was used to make the injections. In most cases the mantle was not damaged by the drilling. After injection the oysters were allowed to remain out of water for 15 to 24 hours. During this period the oysters were maintained in a constant temperature box at 18° C., except in Experiment

30 in which case they were maintained at room temperature. Three experiments---30, 35, and 48---will be discussed below. All of the experiments were conducted in aquaria receiving circulating, filtered sea water as was previously described in Experiment 28.

Milford oysters from the same group as used in Experiment 28 were used in this experiment. Twenty-five experimental oysters and 24 control oysters were injected on June 11, 1953, and placed in aquaria on the following day. Twenty-five oysters were used in the control aquarium, but later one of them was discovered to be a mud-filled "box". The experiment was discontinued on July 16, 1953.

The results are presented in Fig. 17 and Table 4. The mortality in the control oysters was 33 per cent (8 out of 24) compared to 96 per cent (24 out of 25) in the experimental oysters. As in Experiment 28 the oysters in both the control and experimental aquaria incurred considerable initial mortalities, six in each group. If these mortalities are excluded in computing the percentages, the control oysters showed 11 per cent mortality whereas the experimental oysters showed a mortality of 95 per cent---more than 8.5 times greater than that occurring among the controls. All of the control oysters were free of the parasite while all but one of the experimental oysters, which died within four days, were infected with D. marinum. Furthermore, all of the experimental oysters that died subsequent to the initial mortalities and the lone survivor were acutely infected.

2. Experiment 35.

This experiment was a duplicate of Experiment 30 except that the oysters used were from Redfish Bay, Louisiana; these oysters were from the group used in Experiments 36 and 44. Twenty-five control and 25 experimental oysters were injected on July 16, 1953. At the time of injection two of the control and one of the experimental oysters were found to be "cluckers" (live oysters that have lost their shell liquor); there were no available replacements for these oysters. The next day these oysters were gaping when the oysters were placed in the aquaria. Therefore the experiment was begun with 23 control and 24 experimental oysters. The study was terminated on August 25, 1953. Water temperatures ranged from 28° to 31° C.

The results of the experiment are shown in Fig. 18 and Table 4. All but one of the 24 experimental oysters (96 per cent) died whereas only 6 (26 per cent) of the 23 controls died during the experimental period. Some deaths occurred within four days after the start of the study; these deaths were considered due to culling damage sustained by these thin-shelled oysters. Mortality percentages computed after the exclusion of these early deaths show a 96 per cent mortality for the infected oysters while the uninfected show only 11 per cent---more than 8.5 times less than the mortality among the experimental oysters. All of the control oysters were negative for D. marinum. All of the experimental oysters were heavily infected except one that died three days after the start of the study.

3. Experiment 48.

The oysters used in this "injection" experiment were received from Milford, Connecticut, on August 9, 1953. These oysters were shipped by railway express packed in ice and were in transit three days. This method of shipment resulted in the oysters being received in excellent condition, as indicated by the fact that no early deaths occurred in this experiment. Thirty control and 30 experimental oysters were injected on August 13, 1953, and placed in aquaria the next day. The experiment was brought to a close on September 18, when all but two of the experimental oysters had died. Water temperatures ranged from 25° to 30° C. during the period covered by this study.

The results of the experiment are presented in Fig. 19 and Table 4. The first control oyster died 30 days after injection and the first experimental oyster died 19 days after injection. The mortality shown by the control oysters was 7 per cent (2 out of 30) compared to 93 per cent (28 out of 30) among the experimentals---more than 13 times greater than the mortality incurred among the control oysters. All of the experimental oysters showed heavy infections. For the first time, however, an infection was encountered among the control oysters, one of the 28 survivors showing a moderate to heavy infection. There are several possible explanations, namely, (1) the infective elements may have passed through the filters; (2) infective material may have been accidentally introduced into the aquarium; (3) this oyster may have been infected initially (this is considered unlikely since 65

oysters from this group proved to be negative); and (4) there may have been an error in the labeling of the culture or in the removal of the tissue for examination.

3. Discussion of Transmission and Pathogenicity Studies.

The results of the experimental studies show that all four methods of transmission investigated were successful. Both the "injection" and the "feeding" methods have proved to be extremely useful in investigating experimentally the effects of environmental factors on the development of infections as well as the host-specificity of D. marinum and related parasites. It should be pointed out, however, that in closed aquaria control oysters treated by the "feeding" method tend to survive better than those that are "injected". The "injection" of the tissue mince appears to be mainly responsible for this difference in survival of control oysters since oysters drilled and "injected" with materials other than tissue mince survive well in closed aquaria. The evidence from the infection experiments described shows conclusively that direct transmission of D. marinum can occur. At present this constitutes the only proved method of transmission.

All of the three methods used to infect live oysters can not be directly compared with each other as to speed of the development of infection since the "proximity" experiments were conducted only in the winter and the "injection" experiments only in summer. An indirect comparison, however, shows that the infection developed most

rapidly with the "injection" method and least rapidly with the "proximity" method. In the "feeding" experiment (R3-2) conducted in winter the first acutely infected "gaper" appeared during the ninth week, whereas in the "proximity" experiment (R3-1) 16 weeks elapsed before this occurred. In the "feeding" and "injection" experiments conducted in summer, using filtered water, the rate at which acute infections developed in "injection" experiments (30, 35, and 48) was almost twice the rate occurring in the "feeding" experiments (28 and 44). An examination of Fig. 20 reveals that more than 90 per cent of the oysters infected by the "feeding" method died after 62 days; those infected by "injection" required only 41 days to attain this level of mortality. It may also be noted that all "injected" oysters surviving beyond five days were heavily infected, with just one exception, the exception being a moderately infected "gaper" appearing on the tenth day in Experiment 30. Likewise, in the three "feeding" experiments conducted during the summer, all of the "gapers" occurring after the eighth day of experimentation were heavily infected. It is of further interest to note that, with the exception of the single "gaper" in Experiment 30, no oysters in the summer experiments (28, 30, 35, 36, 44, and 48) showed less than heavy infections unless they were survivors. Five of the eight oysters surviving the three summer "feeding" studies showed moderate or less intense infections.

The data presented show a marked acceleration of the mortality rate of the experimentally infected oysters over that of the uninfected

control oysters. Omitting initial mortality obviously due to other causes, all oysters in the eight experiments considered in this discussion showed a death rate among the experimental oysters that was more than twice as great as that occurring among the controls. In five of the experiments the ratios were considerably higher (6.5 to 13 times greater). Such results clearly indicate that D. marinum is pathogenic for oysters under the conditions of the experiments. It also should be noted that uninfected oysters became infected under natural conditions at a rate that was comparable to the rate occurring under experimental conditions. For example, a heavily infected "gaper" was obtained 18 days after a group of uninfected oysters were placed in Bayou Rigaud. Oysters from the same group showed the first heavily infected "gaper" after 16 days with the "injection" method and after 25 days with the "feeding" method. Contrary to one's expectation that the experimental infection rate is quite exaggerated, these results indicate that the rate is similar to that occurring under natural conditions in an area of high endemicity.

The pathogenicity of D. marinum for oysters may be indicated by calculating the ratio of all acute cases of disease to the number of cases terminating in death (Maxcy, 1951). The case-death ratio may be easily determined for the experiments reported herein since (1) the degree of infection is easily and reasonably accurately ascertained and (2) from extensive studies it is known with a fair degree of accuracy that those cases diagnosed as less than moderate do not usually cause

death whereas heavy infections nearly always result in death. Although an oyster may not die immediately upon developing a heavy infection, it will rarely recover from an infection of such intensity.

The case-death ratio is computed in the following manner: (1) the number of acutely infected "gapers" and survivors are determined to establish the number of developed cases of disease; (2) the number of deaths occurring among the experimental and control oysters during the period of acute disease is determined. The period of acute disease extends from the date of the appearance of the first acutely infected "gaper" to the date of the termination of the experiment. Since the control oysters died of causes other than Dermocystidium infection, it is assumed for the purposes of calculation that during this period an equal number of experimental oysters also died of other causes; (3) the total number of oysters acutely diseased at death minus the number of deaths attributed to other causes gives the number of deaths attributed to Dermocystidium infection; and (4) the number of deaths from infection divided by the number of acute infections developed gives the case-death ratio. As an example, the case-death ratio will be computed for Experiment 44. During the period of acute disease three control oysters died and 22 cases of acute disease developed among the experimental oysters, 21 of which resulted in death. The number of deaths caused by Dermocystidium infection is found to be 18, giving a case-death ratio of 0.818. The case-death ratios for all of the experiments may be found by referring to Tables 3 and 4. Only one of

the ratios was found to be less than 0.500 while five of the ratios exceeded 0.800.

Since death is the bext index of pathogenicity, the high case-death ratios exhibited by the oysters experimentally infected with D. marinum demonstrate the extreme pathogenicity of this parasite under experimental conditions. This index was extremely high in those experiments conducted during the summer when high temperatures and relatively high salinities prevailed.

TABLE 1.

Data on Louisiana and Texas Oysters Used in Experiment R3-1

Date obtained	No. dying during course of expmnt.	No. surviving at end of expmnt.	Source
12-3-52	10	0	Grand Isle, La.
12-10-52	11	0	Grand Isle, La.
12-29-52	4	1	Galveston Bay, Tex.
1-26-53	2	3	Galveston Bay, Tex.
2-5-53	2	1	Grand Isle, La.
2-13-53	4	0	Grand Isle, La.
3-4-53	3	0	Grand Isle, La.
3-19-53	6	1	Grand Isle, La.
3-25-53	2	1	Grand Isle, La.

TABLE 2.

Data on Oysters Used to Prepare Tissue Minces Used in Experiment R3-2

Date of "feeding"	Approx. amount tissue mince per aquarium	Intensity of infection
12-9-52 (Control)	Entire live oyster	Negative
12-9-52 (One experimental)	Entire live oyster	Light to moderate
12-10-52 (Other experimental)	Entire live oyster	Very light
12-13-52 (Both experimental)	1/2 oyster ("gaper")	Light to moderate
1-2-53 (Both experimental)	Entire oyster ("gaper")	Light to moderate
1-13-53 (Both experimental)	1/2 oyster ("gaper")	Heavy
1-22-53 (Both experimental)	1/2 oyster ("gaper")	Heavy

TABLE 3

Summary of Results of Experiments R3-1, R3-2, 36, 28, and 44.

Experiment No.	R3-1	R3-2	36[1]	28	44
Origin of oysters	Milford	Milford	Redfish Bay	Milford	Redfish Bay
No. Exam. prior to experiment	28	28	70	98	70
No. infected prior to experiment	0	0	0	0	0
Date experiment started	12-9-52	12-9-52	7-16-53	6-11-53	7-16-53
Date experiment ended	5-2-53	5-1-53	9-11-53	8-11-53	9-18-53
No. of deaths (No. initially used)					
Expmntl. aquaria	20 (40)	25 (30)	25 (30)	24 (25)	23 (25)
Control aquaria	3 (15)	6 (15)	3 (30)	13 (25)	6 (25)
Per cent mortality					
Expmntl. aquaria	50	83	83	96	92
Control aquaria	20	40	10	52	24
Per cent mortality during period of acute disease[2]					
Expmntl. aquaria	44	77	83	94	91
Control aquaria	8	31	7	37	14
No. of cases of acute disease in expmntl. aquaria	27	20	26	17	22
No. of deaths due to disease	12	10	22	9	18
Case-death ratio	0.444[3]	0.500	0.846	0.530	0.818

[1] Experiments 36-1 and 36-2 combined.

[2] Period of acute disease extends from the appearance of the first acutely infected "gaper" to the termination date.

[3] Since this experiment was arbitrarily stopped when mortality attained 50 per cent in the experimental aquarium this ratio is not significant.

TABLE 4.

Summary of Results of Experiments 30, 35, and 48

	Exper. 30	Exper. 35	Exper. 48
Origin of oysters	Milford	Redfish Bay	Milford
No. exam. before expmnt.	98	70	65
No. infected before expmnt.	0	0	0
Date of expmntl. infection	6-10-53	7-16-53	8-13-53
Date experiment ended	7-16-53	8-25-53	9-18-53
No. of deaths (No. initially used)			
In experimental aquaria	24 (25)	23 (24)	28 (30)
In control aquaria	8 (24)	6 (23)	2 (30)
Per cent mortality			
In experimental aquaria	96	96	93
In control aquaria	33	26	7
Per cent mortality during period of acute disease			
In experimental aquaria	95	96	93
In control aquaria	11	11	7
No. cases of acute disease			
In experimental aquaria	19	23	30
No. dead due to disease	16	20	26
Case-death ratio	0.842	0.870	0.867

Figure 9. Results of Experiment R3-1 graphically illustrated. Each block within the outline represents a "gaper" and its position indicates the relative time of death. Shading of the block indicates the intensity of infection, or lack of infection, according to the legend in upper left hand corner. Surviving oysters are represented by blocks placed outside of the outline. Experiment was terminated when 50 per cent of the Milford experimental oysters died. Both graphs of experimental oysters represent the combined results of the four experimental aquaria.

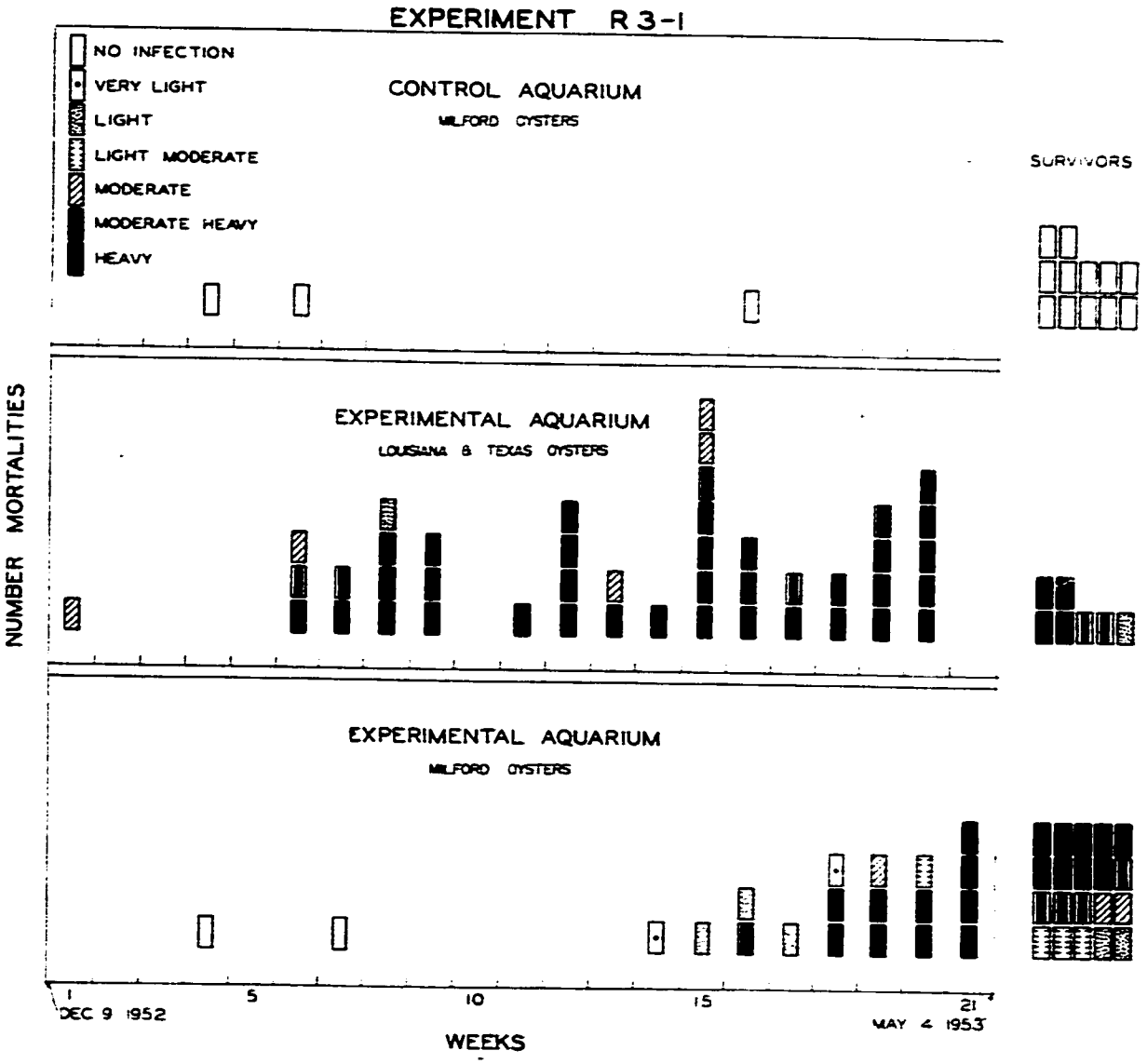

EXPERIMENT R 3-1
NO INFECTION
VERY LIGHT
LIGHT
LIGHT MODERATE
MODERATE
MODERATE HEAVY
HEAVY
CONTROL AQUARIUM
MILFORD OYSTERS
SURVIVORS
EXPERIMENTAL AQUARIUM
LOUISIANA & TEXAS OYSTERS
EXPERIMENTAL AQUARIUM
MILFORD OYSTERS
NUMBER MORTALITIES
1
DEC 9 1952
5
10
15
21
MAY 4 1953
WEEKS

FIGURE 9.

Figure 10. Results of Experiment R3-2 graphically illustrated. See Fig. 9 for explanation of the symbols. The salinity and temperature data also apply to Experiment R3-1. The graph of experimental oysters represents the results of the two experimental aquaria.

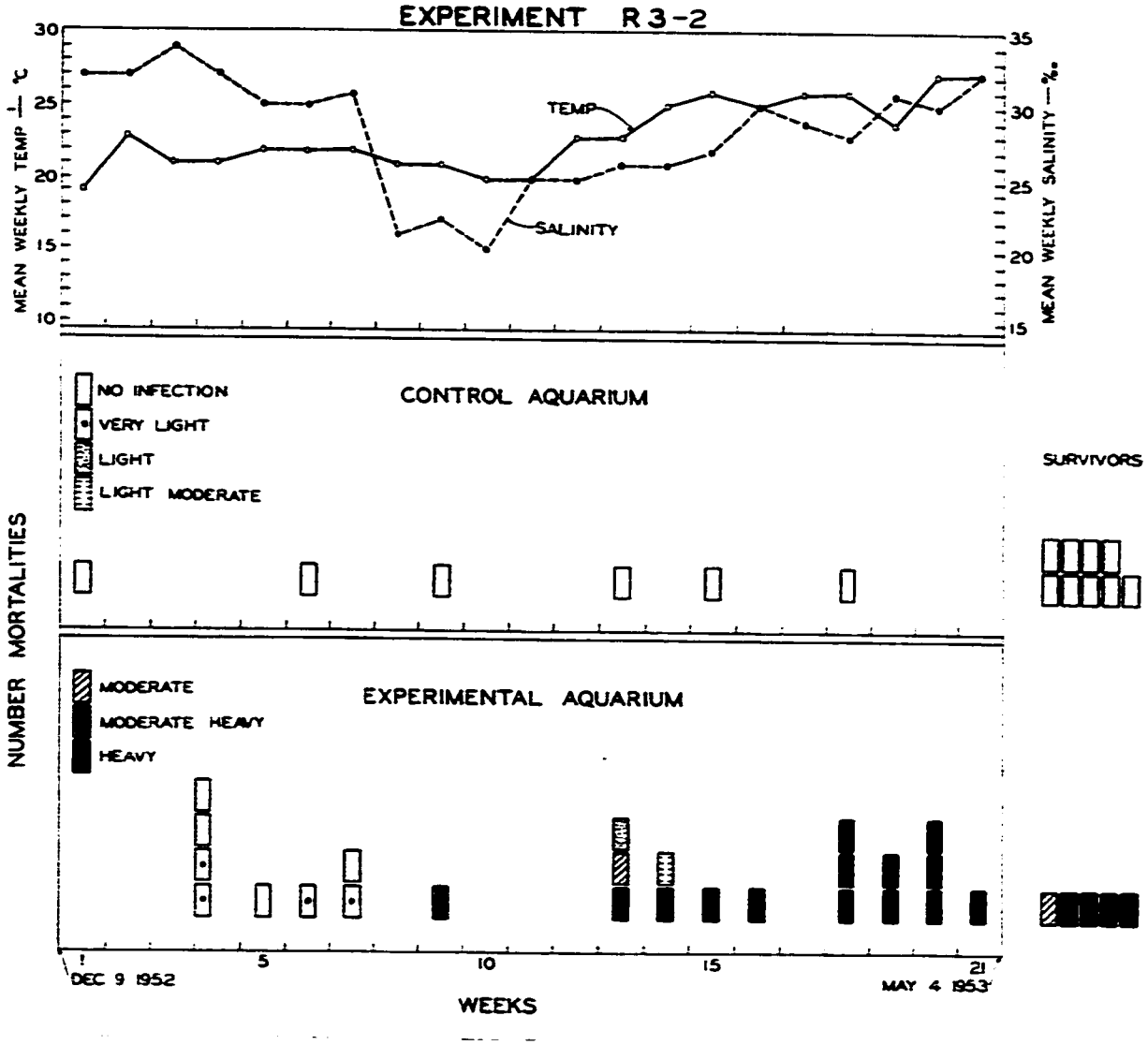

EXPERIMENT R 3-2
MEAN WEEKLY TEMP — °C
MEAN WEEKLY SALINITY — ‰
TEMP
SALINITY
CONTROL AQUARIUM
NO INFECTION
VERY LIGHT
LIGHT
LIGHT MODERATE
SURVIVORS
NUMBER MORTALITIES
EXPERIMENTAL AQUARIUM
MODERATE
MODERATE HEAVY
HEAVY
DEC 9 1952
MAY 4 1953
WEEKS

FIGURE 10.

Figure 11. Results of Experiment 36-1 graphically illustrated. See Fig. 9 for explanation of the symbols. Master control aquarium received no tissue mince; control aquarium received uninfected tissue mince; and experimental aquarium received infected tissue mince.

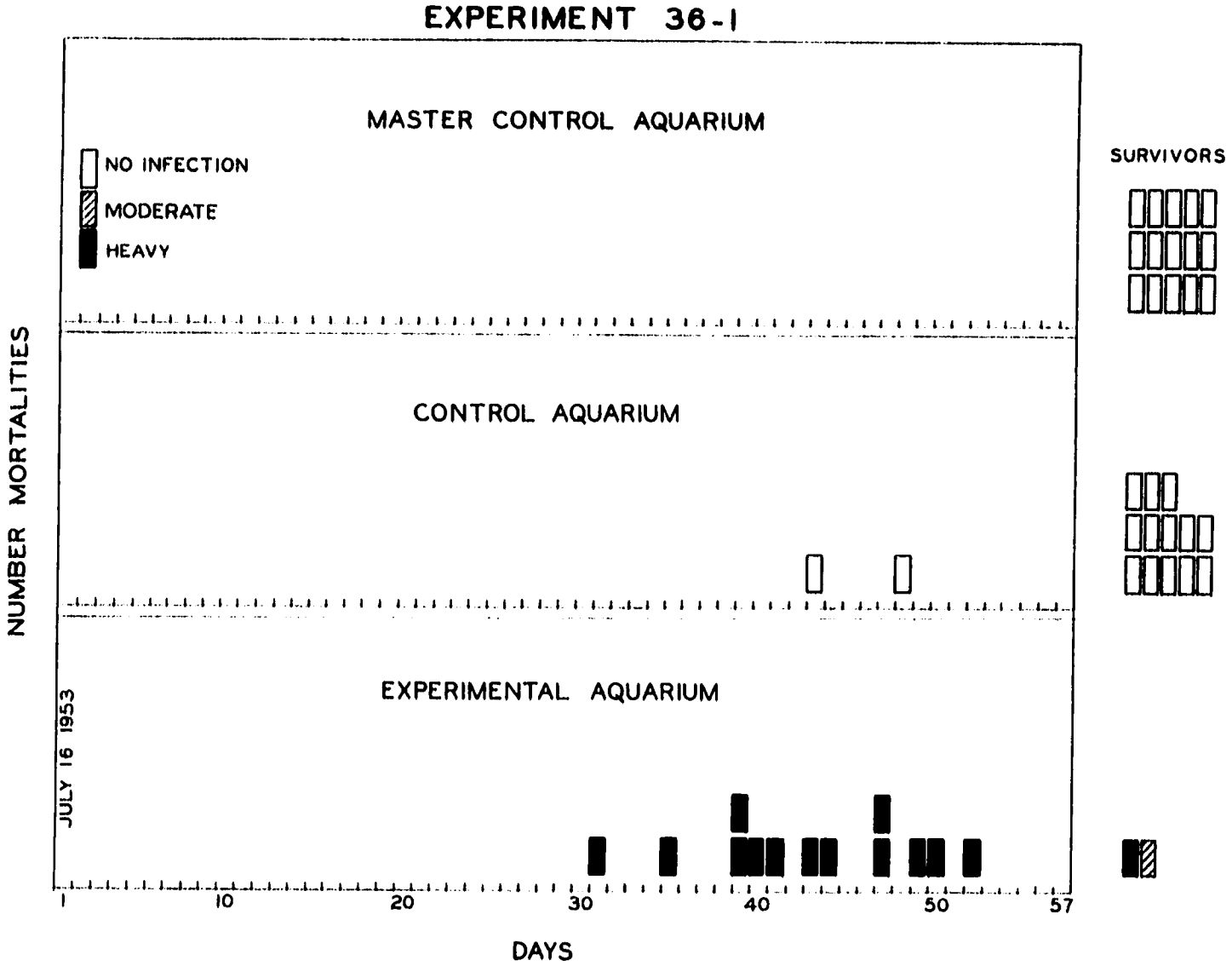

EXPERIMENT 36-1
MASTER CONTROL AQUARIUM
NO INFECTION
MODERATE
HEAVY
SURVIVORS
CONTROL AQUARIUM
EXPERIMENTAL AQUARIUM
NUMBER MORTALITIES
JULY 16 1953
1
10
20
30
40
50
57
DAYS

FIGURE 11.

Figure 12. Results of Experiment 36-2 graphically illustrated. This experiment was a duplicate of Experiment 36-1 (Fig. 11).

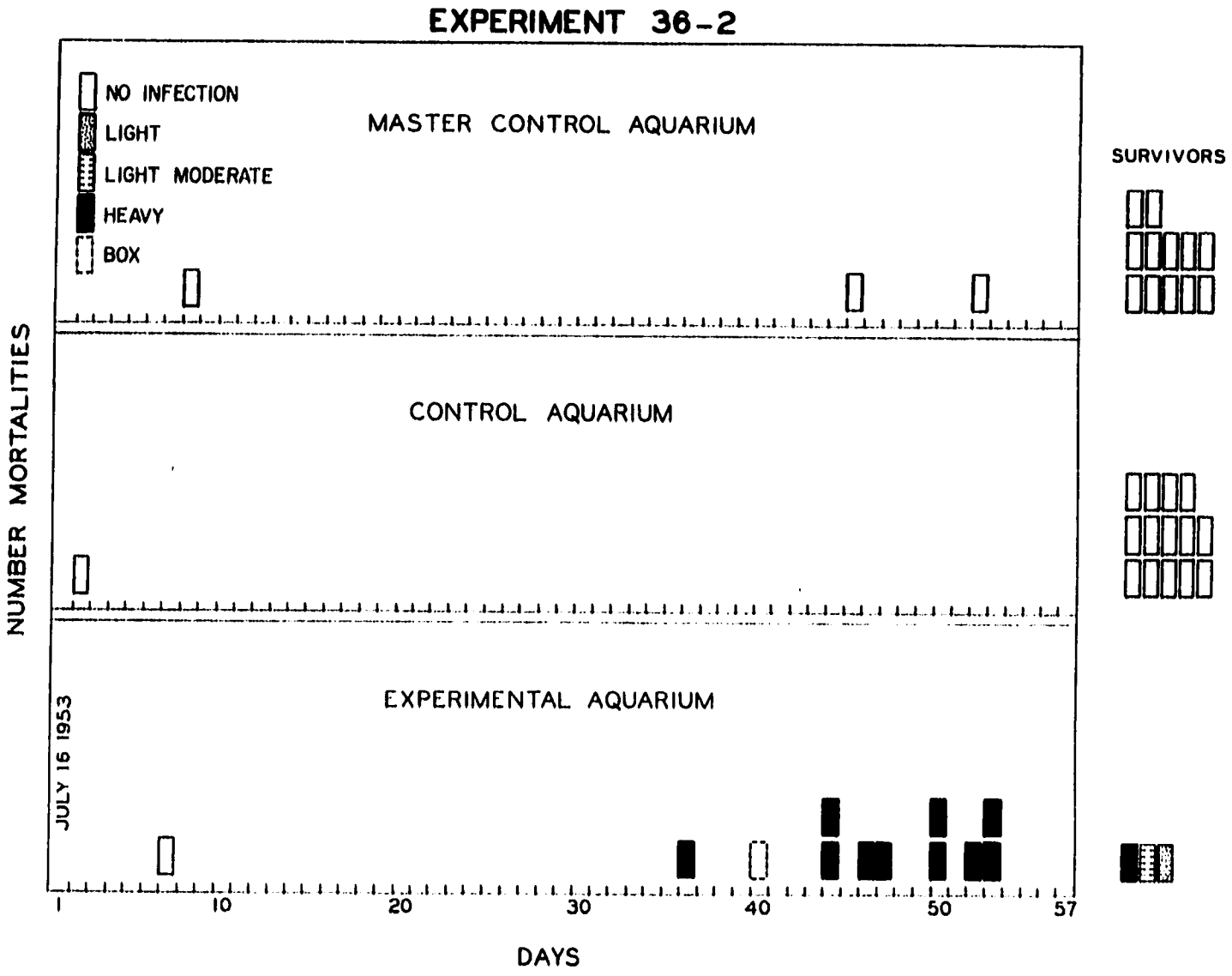

EXPERIMENT 36-2
NO INFECTION
LIGHT
LIGHT MODERATE
HEAVY
BOX
MASTER CONTROL AQUARIUM
CONTROL AQUARIUM
EXPERIMENTAL AQUARIUM
SURVIVORS
NUMBER MORTALITIES
JULY 16 1953
1
10
20
30
40
50
57
DAYS

FIGURE 12.

Figure 13. Results of Experiment 28 graphically illustrated.

See Fig. 9 for explanation of symbols.

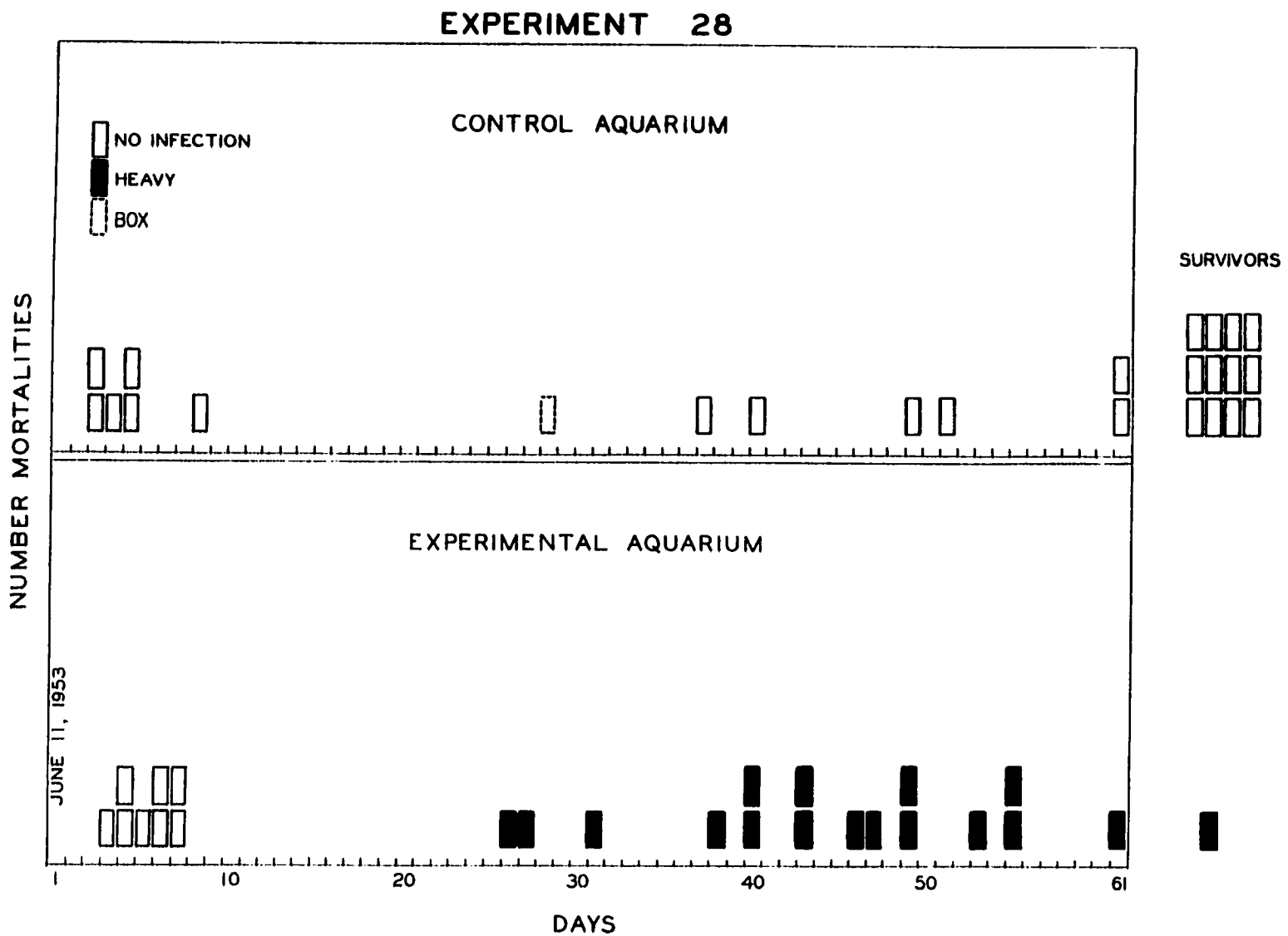

EXPERIMENT 28
CONTROL AQUARIUM
NO INFECTION
HEAVY
BOX
SURVIVORS
NUMBER MORTALITIES
EXPERIMENTAL AQUARIUM
JUNE 11, 1953
1
10
20
30
40
50
61
DAYS

FIGURE 13.

Figure 14. Results of Experiment 44 graphically illustrated.

See Fig. 9 for explanation of symbols.

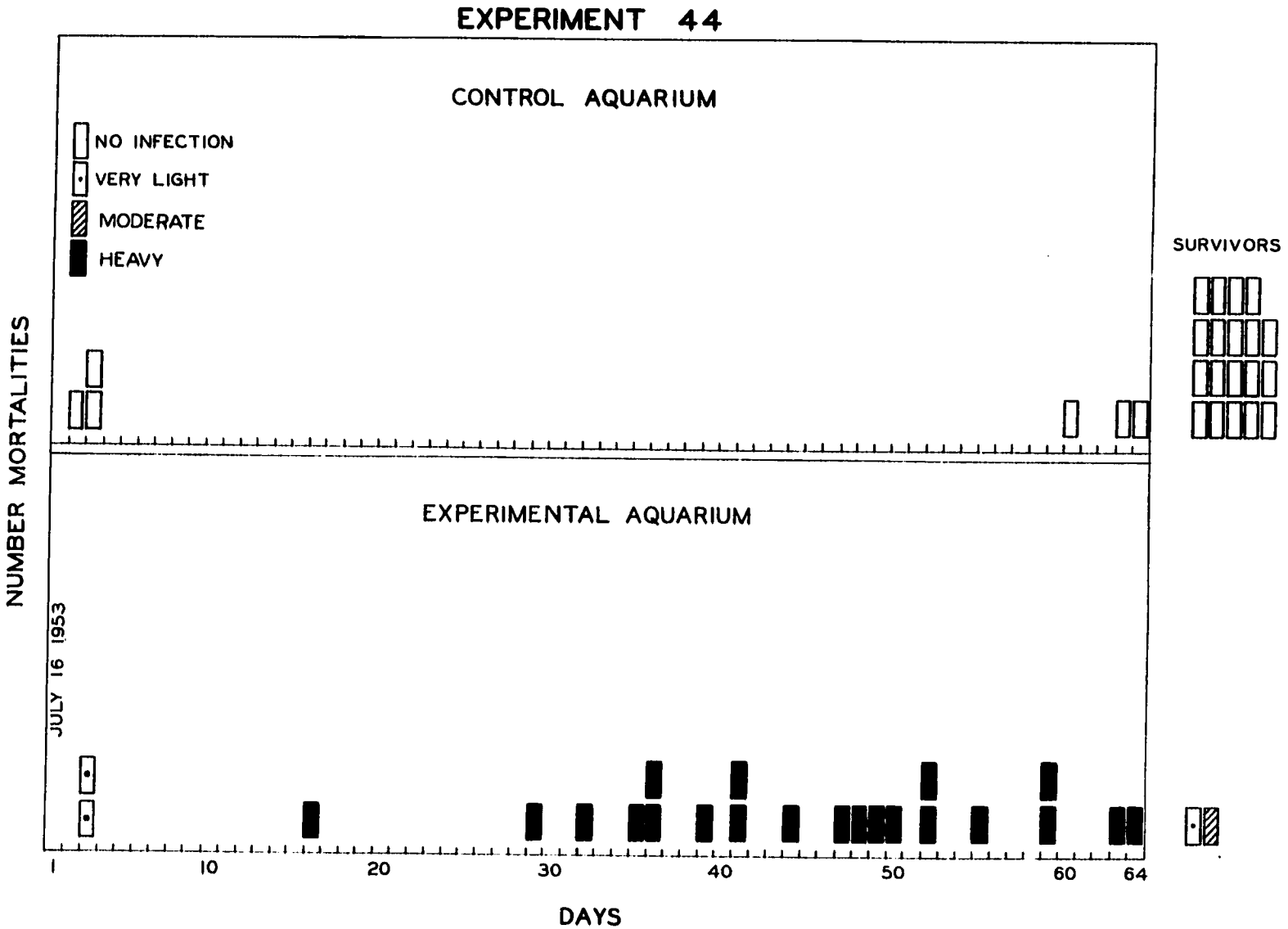

EXPERIMENT 44
CONTROL AQUARIUM
NO INFECTION
VERY LIGHT
MODERATE
HEAVY
SURVIVORS
NUMBER MORTALITIES
EXPERIMENTAL AQUARIUM
JULY 16 1953
1
10
20
30
40
50
60
64
DAYS

FIGURE 14.

Figure 15. Photograph of the apparatus used to filter the Bayou Rigaud sea water used in Experiments 28, 30, 35, 44, and 48. Three sets of filters and aquaria are shown. The three aquaria above serve as settling and constant head tanks. Below these are three containers in tandem, each containing thick pads of glass wool. The water from each set of filters is divided equally between the control aquarium (in the foreground) and the experimental aquarium behind.

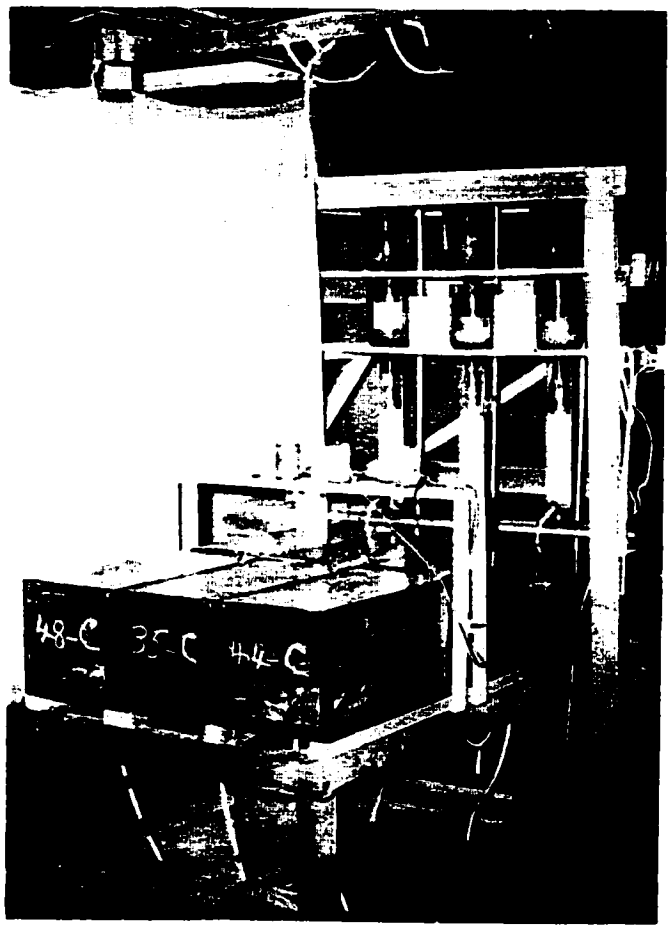


FIGURE 15.

Figure 16. An oyster showing the relative position in which the holes were drilled for the "injection" of tissue mince into the mantle cavity.

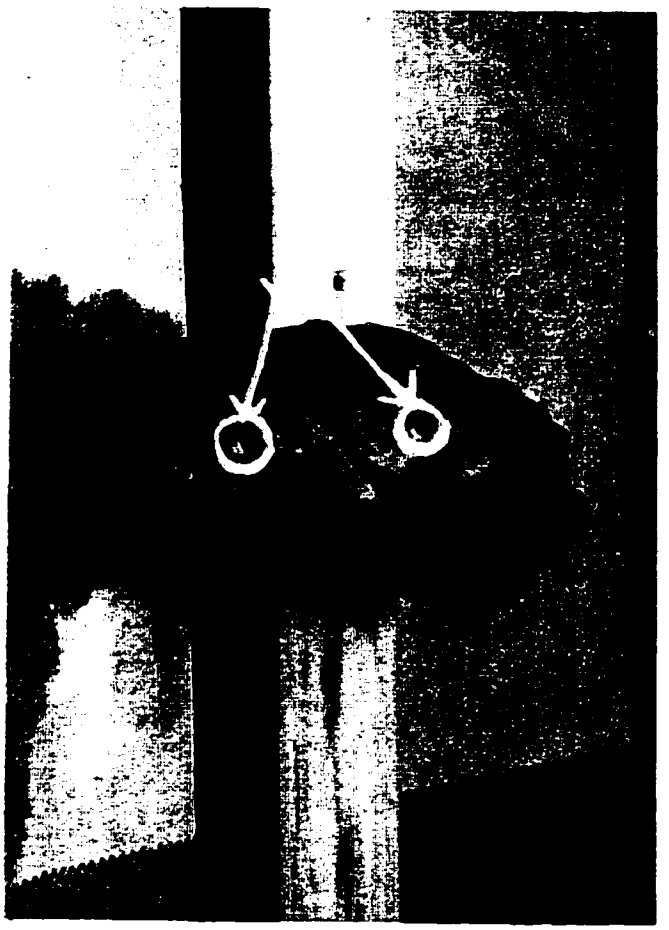


FIGURE 16.

Figure 17. Results of Experiment 30 graphically illustrated. Experiment was started June 11 instead of June 10 as indicated in figure. See Fig. 9 for explanation of symbols.

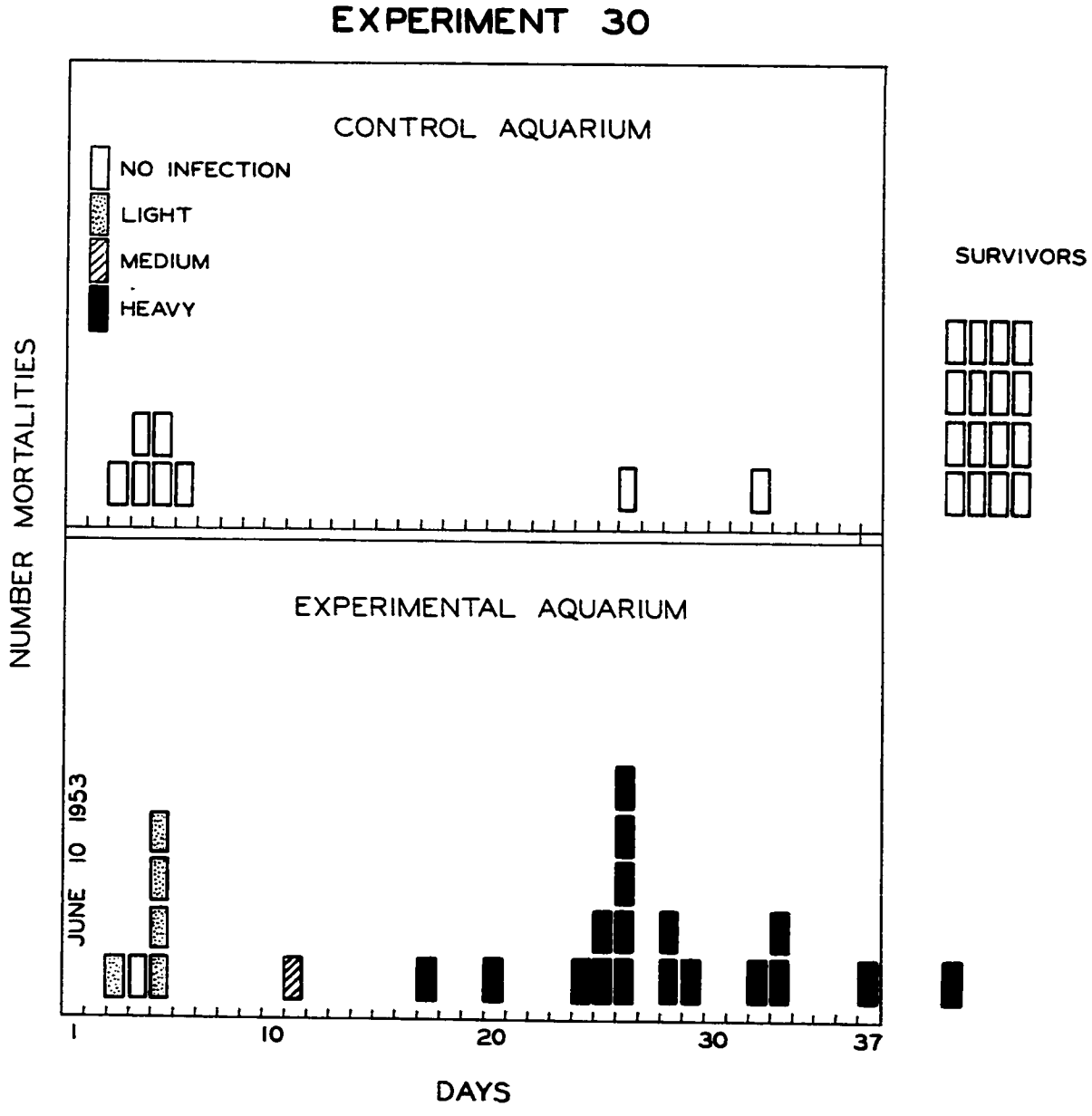

EXPERIMENT 30
CONTROL AQUARIUM
NO INFECTION
LIGHT
MEDIUM
HEAVY
SURVIVORS
NUMBER MORTALITIES
EXPERIMENTAL AQUARIUM
JUNE 10 1953
1
10
20
30
37
DAYS

FIGURE 17.

Figure 18. Results of Experiment 35 graphically illustrated.

See Fig. 9 for explanation of symbols.

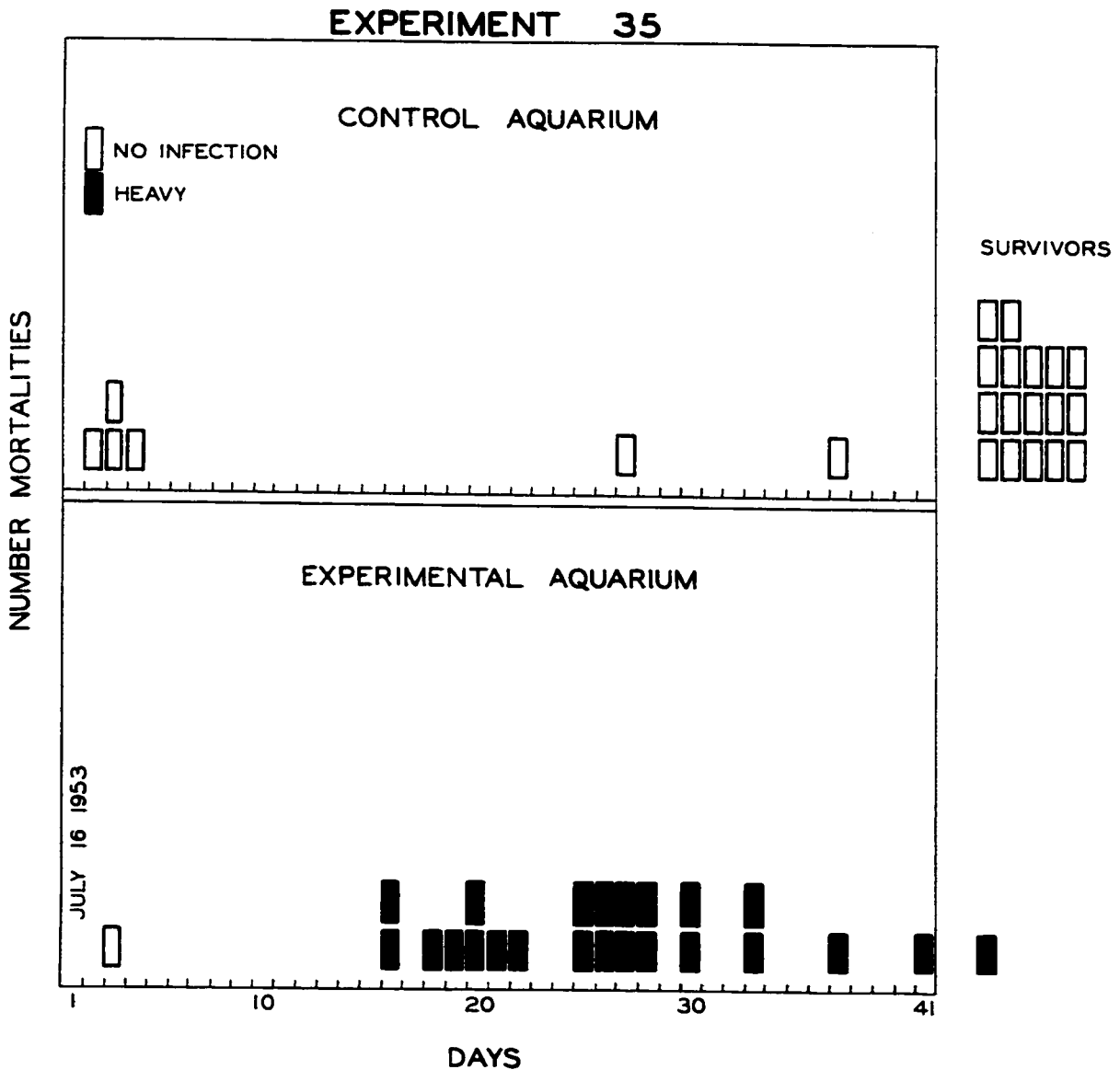

EXPERIMENT 35
CONTROL AQUARIUM
NO INFECTION
HEAVY
SURVIVORS
NUMBER MORTALITIES
EXPERIMENTAL AQUARIUM
JULY 16 1953
1
10
20
30
41
DAYS

FIGURE 18.

Figure 19. Results of Experiment 48 graphically illustrated.

See Fig. 9 for explanation of symbols.

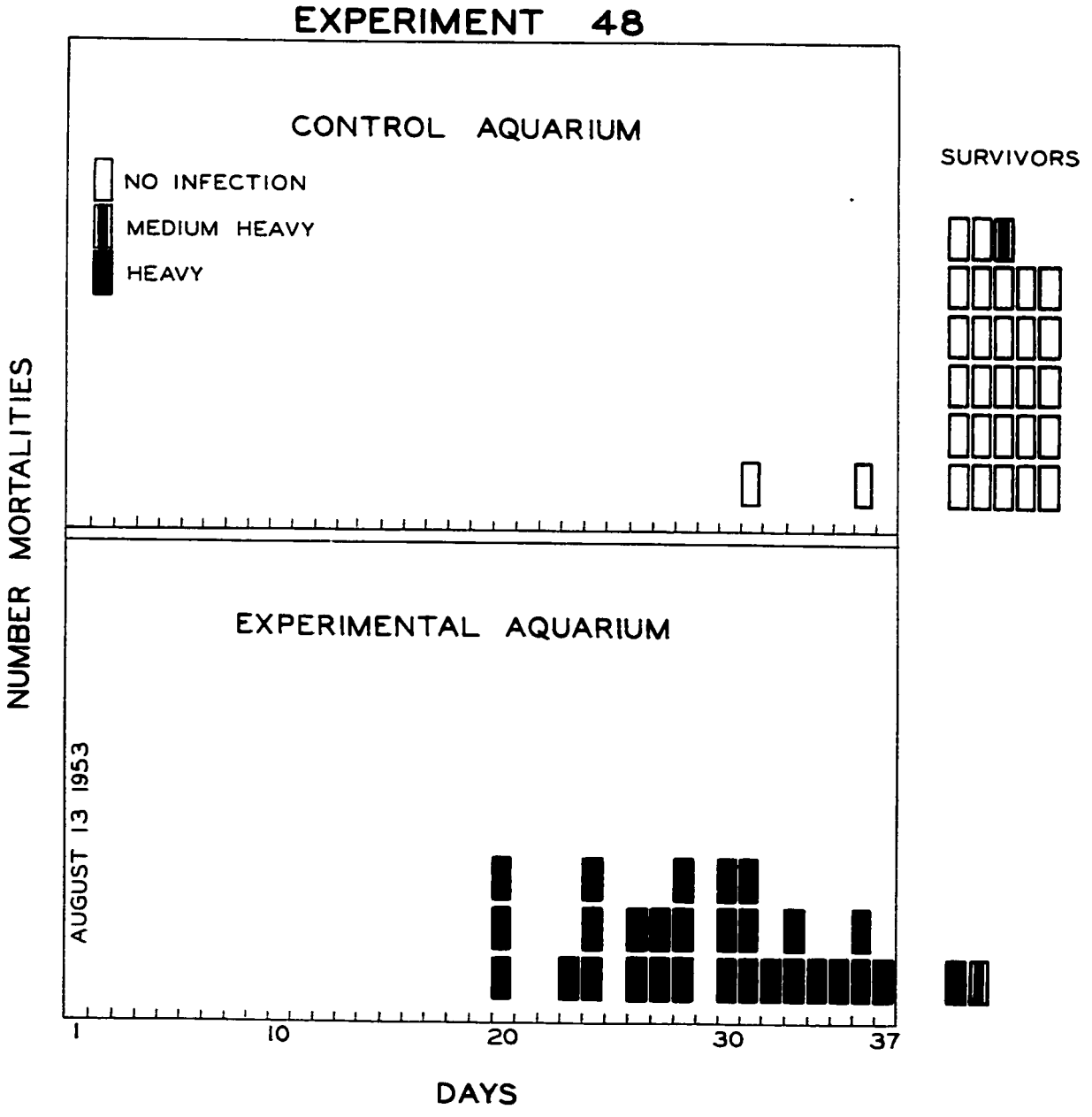

EXPERIMENT 48
CONTROL AQUARIUM
NO INFECTION
MEDIUM HEAVY
HEAVY
SURVIVORS
NUMBER MORTALITIES
EXPERIMENTAL AQUARIUM
AUGUST 13 1953
1
10
20
30
37
DAYS

FIGURE 19.

Figure 20. A graphic comparison of the results of infection by "feeding", Experiments 28 and 44 combined, with those by "injection", Experiments 30, 35, and 48 combined. See Fig. 9 for explanation of symbols. Note: as a result of a slight error in Fig. 17 a few of the blocks in the composite graph of Experiments 30, 35, and 48 were shifted either one day ahead or one day behind the actual date of death. The block placed at 40 days should have been placed at 41 days.

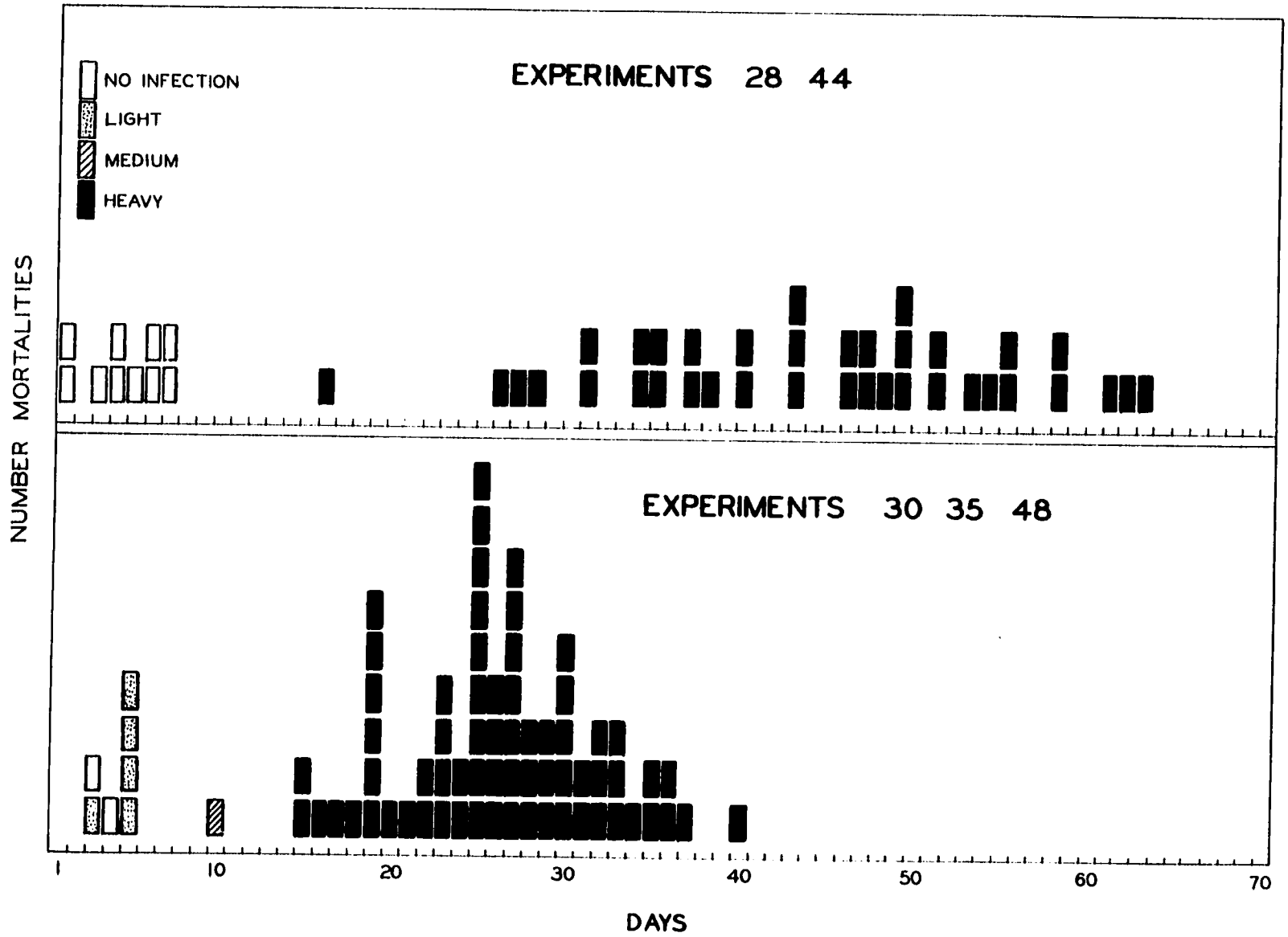

NO INFECTION
LIGHT
MEDIUM
HEAVY
EXPERIMENTS 28 44
EXPERIMENTS 30 35 48
NUMBER MORTALITIES
1
10
20
30
40
50
60
70
DAYS

FIGURE 20.

PATHOLOGICAL STUDIES

Most oysters heavily parasitized with D. marinum are characterized grossly by a more or less marked shrinkage of the entire body which, therefore, occupies less of the shell cavity than does the body of a parasite-free or lightly infected oyster. The most extensive shrinkage usually occurs in the visceral mass. An occasional heavily infected "gaper" is recovered which does not differ appreciably in appearance from normal oysters except that the gill and mantle tissues are usually noticeably thinner. The heart is often filled with pus (masses of parasites and leucocytes) and easily visible yellowish abscesses are sometimes seen on the visceral mass.

Mackin (1951a) studied extensively the histological changes occurring in oysters infected with D. marinum. He found that, (1) any tissue may be invaded, although external epithelia and peripheral nerves are not usually invaded; (2) infection becomes massive in high salinity waters during high temperature months; and (3) damage to host tissues is largely caused by lytic action of the parasites coupled with embolism in later stages. He concludes that the parasites apparently produce no toxic effects since extreme concentrations of parasites may be built up before death of the host; he also considers that circulatory failure is probably one of the most common immediate causes of death. Additional studies on the pathological effects of D. marinum are presented below.

1. In Vitro Histolytic Studies.

During the course of cultural studies previously considered, it was discovered that small, living pieces of heavily infected tissues maintained in sterile sea water fortified with antibiotics tended to disintegrate much more rapidly than did negative or lightly infected tissues. Small pieces of tissue have maintained strong ciliary activity in vitro for as long as 30 or 40 days without an appreciable change in gross appearance when negative or only lightly infected. Those tissues heavily infected, however, began to show a progressive disintegration after about a week in spite of the fact that bacterial decomposition was retarded by antibiotics. Although the disintegrating tissues maintained ciliary activity, the intercellular cementing substance appeared to be destroyed by the parasites, which increased considerably in these cultures. The ciliated tissues as well as others became disarticulated into numerous individual cells and aggregates of cells. The individual ciliated cells and the aggregates of such cells moved about actively in the sea water. Initially the heavily infected tissues showed some shrinkage, but the disintegration was not noticeable unless the tissues were disturbed. Since attempts to remove such tissues caused them to fragment, it was practically impossible to pick them up with forceps. Uninfected or lightly infected tissues, however, could be removed without fragmenting appreciably for many weeks after ciliary activity had ceased.

These observations formed the basis of attempts, made in collaboration with J. G. Mackin and J. L. Boswell (Ray, Mackin, and Boswell,

1953), to measure quantitatively the histolytic effects of D. marinum on oysters and the consequent loss of weight.

Small pieces of gill tissue were excised from "gapers" (experimental) and live oysters (control) from the same source; these tissues were placed in sterile Carrel tissue culture flasks containing a small amount of sterile sea water. The sea water was fortified with penicillin and streptomycin (about 500 units of each per ml.) to retard bacterial development. All gill tissues used showed vigorous ciliary activity. After removal from the shell and prior to excisement of the pieces of tissue, each oyster was (1) washed thoroughly with sterile sea water, (2) washed with a solution of sodium merthiolate (1 to 10,000) for about 10 minutes, (3) washed again in sterile sea water, and (4) allowed to remain in sterile sea water fortified with 1000 units of each of the antibiotics for several hours. This treatment was performed to reduce the contaminants on the oyster. Small pieces of gill tissue were then placed in Carrel flasks containing sea water fortified with antibiotics. The neck was plugged with sterile cotton held in place by a small rubber sleeve. In spite of these precautions contamination occurred in the majority of the cultures; most of the experiments were discarded because of the appearance of contaminating molds, yeast, or ciliates. The latter were particularly troublesome since they were apparently not affected by the antibiotics. However, a sufficient number of cultures remained free of contamination to permit us to make comparative studies. Only those control tissues that proved to be completely free of D. marinum were used in

comparing the degree of disintegration occurring in the experimental and control tissues.

Six experimental and six control flasks were set up in each unit study. Photographs were made at the beginning of each study, again at an intermediate stage, and at the end of the study. These photographs recorded the progress of the disintegration of the tissues. The contents of all flasks were examined microscopically at the end of each study to determine if contaminating organisms were present. The results of one set of the studies of lysis of excised tissues are illustrated in Fig. 21 (photograph taken at start of experiment on July 17, 1952), Fig. 22 (taken a week later) and Fig. 23 (taken on July 13, 1952), at which time the experiment was terminated.

The results are obvious from the photographs. The tissues parasitized by D. marinum were completely disintegrated in all six experimental flasks. A few active ciliated cells from the gill epithelium were found in the experimental flasks at the end of the study; aside from these, little material identifiable as originally part of the gill tissue remained. The granular debris left consisted almost entirely of masses of Dermocystidium cells. A photomicrograph of these is shown in Fig. 24. The spheres are cells of D. marinum. One experimental Carrel flask culture (bottom, first row in Fig. 23) was contaminated by a mold; all others were free of contaminants. The controls were free of contaminants except the tissue in flask number two of the last row; this culture was contaminated by a mold. All of the tissues were still intact and showing

ciliary activity, even the piece contaminated with the mold. The cloudiness of the control flasks in Fig. 22 was due to diapedesis of masses of leucocytes which moved out of the tissues into the sea water. Only a few of these were in the experimental flasks since most leucocytes are destroyed by the parasites in heavily infected oysters. The shrinkage of the control tissues was due to autolysis and loss of leucocytes by diapedesis. Just before the photograph, Fig. 23, was taken, the leucocytes were removed with a pipette so that the flasks would not be clouded as in Fig. 22. After this photograph was taken, the cultures were examined and discarded.

The above studies confirm Mackin's (1951a) conclusion that a major effect of Dermocystidium disease is lysis of host tissues. The rapid dissolution of the parasitized tissues compared to the very slow disintegration of uninfected control tissues indicates that D. marinum produces a lytic enzyme. Furthermore, observations suggest that an important action of the parasite is the destruction of the intercellular cementing substances.

2. Quantitative Measurement of Reduction in Meat Weight.

This investigation was conducted at Grand Isle, Louisiana, and the work was done principally by J. G. Mackin and J. L. Boswell. This study and the in vitro histolytic studies discussed above were combined for publication (Ray, Mackin, Boswell, l. c.). The following is a summary of the methods used and the results obtained.

As previously mentioned, oysters heavily parasitized with D. marinum generally show a considerable shrinkage of the entire meat. The purpose of the study was to determine the relationship between the wet weight of the meat and the volume of the shell cavity of the oysters in various stages of Dermocystidium disease. The procedure was to collect daily the "gapers" from the trays containing naturally infected oysters. For each "gaper" used, a living oyster was selected randomly as a control; only those "gapers" which showed evidence of being alive were used. The wet weight and the volume of the shell cavity were determined for each oyster. The intensity of infection was determined for each oyster by use of the thioglycollate culture technique. This study was begun on April 4, 1952, and was terminated on August 30, 1952.

During the period of the study, 176 "gapers" and 22 live oysters were found heavily infected---a total of 198; 83 were moderately infected; and 227 oysters were either uninfected or lightly infected. Since the lightly infected oysters showed a mean weight per unit of shell capacity (inside volume of the closed shell) of only eight to nine per cent less than uninfected oysters, these two groups were combined for the purposes of analysis. The negative-light class was used as the control group in ascertaining the relative weight loss of moderately and heavily infected oysters. The average wet weight of the meats was reduced to weights per unit of shell capacity. The moderately infected oysters showed an average loss of weight of about 12 per cent in early spring and about 15

per cent during the latter part of August. Those heavily infected lost from 20 per cent (in cool months) to 50 per cent (in summer) of the total meat weight, an average loss of about 33 per cent.

These results show conclusively that acute infection with D. marinum causes appreciable loss of weight in oysters. Evidence indicates that the lytic action of the parasites is primarily responsible for the loss of weight; starvation may a lso be an important factor since the intestinal epithelium is extensively damaged by this parasite. Histological studies performed by Mackin show that heavily infected oysters do not feed in the late stages of disease; feeding does take place while they are moderately or lightly infected. Since ingestion of food, however, does not necessarily mean that digestion and assimilation continue in a normal manner, weight loss due to starvation may begin to some extent during the early stages of the disease. In endemic areas the loss of meat weight results in an important economic loss that must be borne by the oyster industry in addition to the much greater loss accruing from mortality.

Figure 21. Photograph of a unit study of lysis of excised gill tissues, made July 17, 1952, when the study was initiated. Carrel flasks in vertical rows marked "E" contain gill tissues heavily infected with D. marinum; those marked "C" are uninfected controls.

Figure 22. Photograph of the Carrel flask lysis study shown in Fig. 21, taken on July 23, 1952. The cloudiness of the controls was caused by diapedesis of leucocytes.

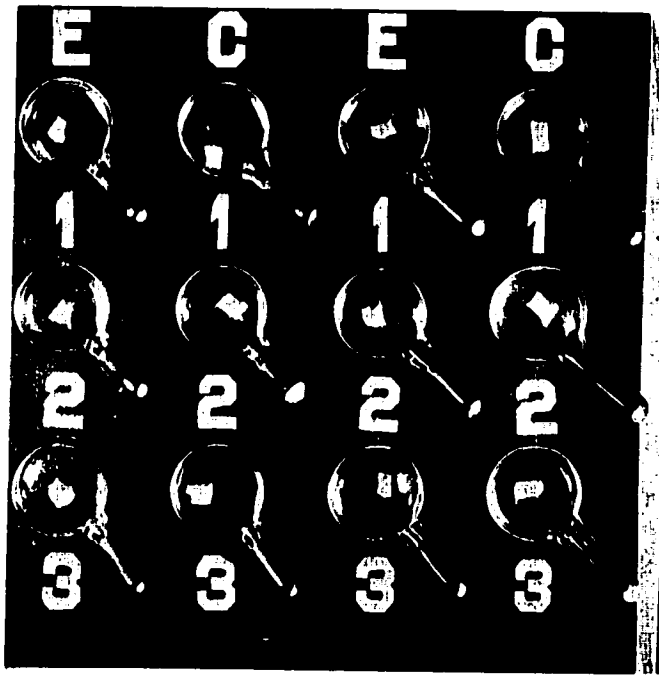


FIGURE 21.

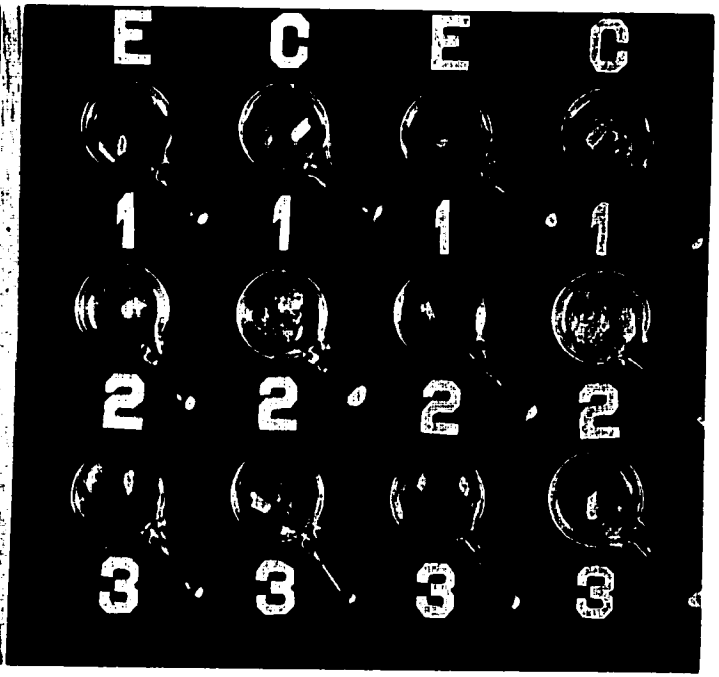


FIGURE 22.

Figure 23. Photograph of the Carrel flask lysis study shown in Figs. 21 and 22, taken on July 31, 1952, when the study was concluded. Note the almost complete destruction of parasitized tissue. Shrinkage of controls was caused by diapedesis of leucocytes and by autolysis. The third flask in the first row of experimental tissues and the second flask in the last row of control tissues were contaminated by a mold. Note: the flasks in the first and last rows are not as clear in reproduction as they were in the original photograph.

Figure 24. Photomicrograph of a small piece of debris taken from one of the experimental Carrel flasks (the first one of the left hand row). The original tissue was almost completely replaced by Dermocystidium cells, the minute spheres making up most of the fragment; unstained.

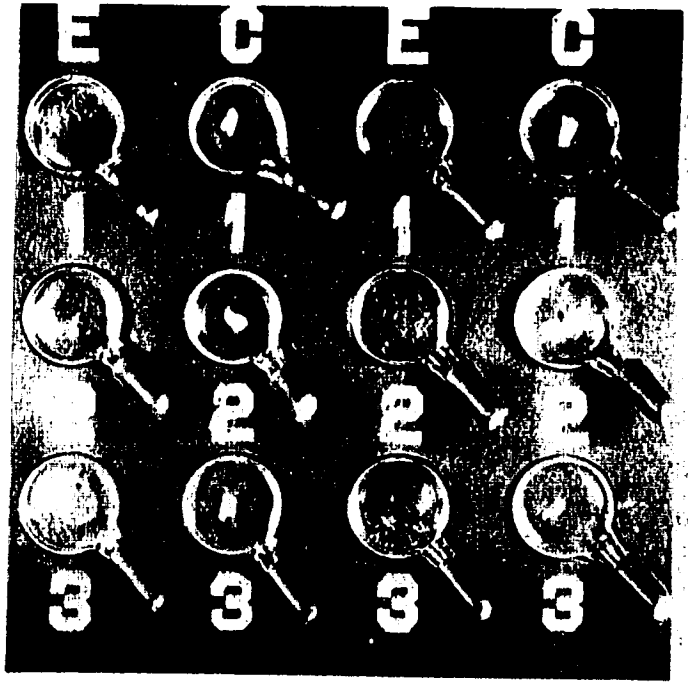


FIGURE 23.

FIGURE 24.

STUDIES ON THE OCCURRENCE OF D. MARINUM IN OYSTERS OF VARIOUS AGES

This investigation (Ray, 1953) was conducted to determine the relative degree of susceptibility of oysters of various ages, especially those under a year old, to Dermocystidium infection under natural conditions. Mackin (1951a) found that younger oysters are not infected as extensively as are the older market size oysters. In the presently considered studies oysters were examined for the presence of the parasite at various periods beginning shortly after setting and extending approximately one year. Two groups of these young oysters were used. In addition, two other groups of oysters of more advanced age were studied in order to compare the rate of infection of older oysters. One of these older groups was approximately one year old at the beginning of the study and the other group was composed of oysters four or more years old.

On June 11, 1952, six bags of clean oyster shells were put out at each of two stations: Sugar House Station (extreme lower Barataria Bay, Louisiana) and Station 51 (middle lower Barataria Bay, Louisiana). These shells were taken up on June 25 and June 26, 1952. The shells bearing spat were separated and each spat (usually one, rarely up to three per shell) was marked on the right valve with finger nail polish. The marked spat were placed in bags of galvanized chicken wire and suspended from the dock of the Texas A. & M. Research Foundation Laboratory located over Bayou Rigaud, Grand Isle, Louisiana. The

bottoms of these bags were suspended approximately one foot above the water bottom. This group of spat was designated as Group A.

On June 12, 1952, a large number of spat (estimated age, one to three weeks) were found on "boxes" in some of the storage trays in Bayou Rigaud. These spat were treated in the same manner as those described above. This second group of spat was designated as Group B. Spat that subsequently became attached to the shells in these bags were periodically removed to avoid confusion, and the marked spat were occasionally re-marked to insure proper identification. The bags containing both groups of spat were located within six to eight feet of several trays of oysters that were at least 75 per cent infected with D. marinum during the summer months.

On June 25, 1952, Mackin removed a tray of approximately one year old oysters from the storage racks in Bay Chene Fleur, Louisiana, (a low salinity and low mortality area) and placed it on the storage racks in Bayou Rigaud. These oysters were caught as spat in Sugar House Bayou between June 21 and July 23, 1951. They were maintained in Bay Chene Fleur from July 24, 1951, until June 25, 1952. This group of oysters was designated as 11 to 12 month old oysters. In view of the fact that oysters approximately a year old were known to be susceptible to D. marinum, this group served as a control in this investigation. Twenty-five of these oysters were examined on June 25, 1952, and all were negative. This indicates that the level of infection was very low. However, a "gaper" which was heavily infected was re-

covered from this group on the same day, indicating that there were a few infected oysters.

The fourth group of oysters, estimated to be four years old, was obtained from Gardiner's Bay, Long Island, New York, on August 21, 1952. These oysters were used to determine the time required for oysters from a non-endemic area to become infected when placed in endemic waters. These oysters were maintained in trays on the tide gauge racks in Bayou Rigaud. Thirty of these oysters were examined on August 22, 1952, and all were found to be negative for D. marinum.

In most cases the diagnoses were based on the examination of rectal tissue after 48 to 72 hours of incubation in thioglycollate medium. The Groups A and B spat were cultured in entirety through August 22, 1952. After this date rectal tissue and a part of the visceral mass were cultured for examination. At the time of culturing the length of each of the spat from Groups A and B was measured to the nearest millimeter.

The results of the examinations for D. marinum, as well as other pertinent data, are recorded in Figs. 25 and 26 and Tables 5, 6, 7, and 8. Figure 25 was prepared by plotting the incidence of infection in the three different age groups studied, against time in months. Figure 26 was prepared by plotting the weighted incidence in the three groups against time in months.

An inspection of Tables 5 and 6 shows that the incidence and intensity of D. marinum in young oysters is extremely low (incidence less than one per cent) during the first three to four months after setting. Only two individuals were found to be infected out of 275 spat that were checked between June 12 and September 19, 1952. One of these two cases of sporadic infection was found in a spat six to eight weeks old and the other was found in a spat nine to ten weeks old. Because of the paucity of data obtained during October and November and the fact that lower water temperatures during the fall and winter months bring about a decrease in the infection rate of D. marinum, no conclusions can be drawn at the present time as to whether or not this low degree of susceptibility continues for a longer period. Perhaps the high degree of refractivity to this fungous parasite is due to an "age immunity". If this be true, however, it is the reverse of the usual situation, for in all other cases of "age immunity" to animal parasites resistance increases with advancing age after the brief period when, in mammalian hosts, the offspring are passively protected by anti-bodies acquired from the mother. Other possibilities are (1) that this low infection rate may be due to some factors connected with the feeding of the host and/or the life cycle of the parasite; (2) that the phagocytes, which constitute an important defense mechanism in invertebrates, may be more effective in resisting the invasion of D. marinum in young than in older hosts; (3) that the high metabolic activity of the rapidly growing spat may contribute considerably to this apparent "age immunity".

Further examination of Table 5 shows a more or less gradual increase in the incidence and intensity of infection from November 28, 1952, to June 13, 1953. One oyster was found to be infected out of 15 examined at 23 to 24 weeks, and another one out of the same number was found to be infected at 33 to 34 weeks. At 41 to 42 weeks two of the oysters out of 15 examined proved to be infected. It is evident from data presented in Tables 5 and 6 that the susceptibility of these oysters was significantly increased approximately one year after setting. At this age 30 of the 92 individuals checked (33 per cent) were diagnosed as positive for D. marinum. The weighted incidence for this group of oysters at this time was 0.6.

The growth of the spat in Groups A and B as indicated by the total lengths was very good. At approximately one year of age the majority of these oysters were well shaped and rather deeply cupped. The oysters in these two groups averaged 81 millimeters in length about one year after setting; the average length was equal to the legal minimum marketable size of three inches.

An examination of Table 7 reveals that oysters 11 to 12 months old became positive for D. marinum after one month in Bayou Rigaud. An examination of Table 8 reveals that the Long Island oysters became infected between the first and third week. There was some mortality among the Long Island oysters within six weeks after initial exposure which was probably caused by D. marinum. Except for the initial mortality, which was certainly due to the effect of transportation, the Long

Island oysters showed a relatively low mortality until the following spring when the weighted incidence reached a relatively high level.

A study of Fig. 26 shows clearly that there was a very significant age differential in the degree of susceptibility among the three age groups considered in this investigation. In the first place, the Groups A and B spat required a year to attain the weighted incidence (0.6) which was reached by the year old oysters within eight weeks. Similarly, one may note that Long Island oysters attained within six weeks a weighted incidence (1.55) which was 50 per cent higher than the maximum weighted incidence (1.0) which occurred in the year old oysters within 12 weeks.

Although this investigation was not set up as a mortality study, some data were obtained on the mortality that occurred in each group except the group of 11 to 12 month old oysters. The mortalities in Groups A and B were not checked until the spat were about one to two inches in length. These spat did not appear to have an appreciable mortality until small conchs (Thais haemostoma) entered the bags. The mortality from conchs greatly decreased as the oysters increased in size. It is assumed that the small conchs were unable to pierce the valves of the larger oysters and also that the activity of the conchs probably diminished during the cool months. The per cent of mortality per day recorded for the Group A spat in Table 5 represents the combined mortality of the spat collected at Sugar House Station and Station 51. Except for the mortality due to conchs, the mortality of the Groups

A and B spat remained at a low level during the entire year. From April 3, to June 6, 1953, the Group B spat showed a mean mortality of 0.08 per cent per day; the Group A spat showed a mean mortality of 0.14 per cent per day from April 3, to June 13, 1953.

The mortality figures given in Table 8 for the Long Island oysters are not exactly accurate in all cases because the mortalities were not determined at each interval that oysters were removed for Dermocystidium examinations. The mortality figures recorded in Table 8 were obtained by taking the average of the per cent mortality derived in two different ways. One value represented the per cent mortality based on all oysters on hand at the beginning of each period, including those that were removed for examination. The other value represented the per cent mortality based on the number of live oysters on hand at the end of the period. The mortality figures for April 3 and June 4, 1953, are accurate. On April 3, 1953, 13 "boxes" and five "gapers" were recovered. This suggests that a large percentage of the mortalities that accumulated between February 5 and April 3, 1953, had occurred very recently, or this number of "gapers" would not have been recovered. Three of these "gapers" were heavily infected and two moderately infected. This sharp rise in incidence and intensity of D. marinum as well as mortality is undoubtedly associated with the rise of water temperatures. Between April 3 and June 4, 1953, several conchs entered the tray containing the Long Island oysters. It is not known how many of the mortalities that occurred in this tray were

caused by the conchs. It is, however, the opinion of the writer that damage from the conchs was negligible. The oysters were encrusted with large numbers of small oysters which the conchs were observed attacking at the time the tray was examined. Of the 50 "boxes" recovered at this time, three bore evidence of crab damage. It is not possible, therefore, to state definitely what part of the mortalities occurring at this time was due to Dermocystidium infections; nevertheless, in view of the extremely high weighted incidence (2.3) among the living oysters at this time, the writer believes that the great majority of the mortalities were caused by D. marinum. It also should be noted that there was a marked decrease in weighted incidence along with a slight decrease in incidence among the Long Island oysters in November, 1952, and January, 1953. This decrease suggests that oysters are able to rid themselves of a large portion of the parasites during the colder months.

Since the young oysters grew and survived well in an area of high mortality for a year, it appears certain that the environmental conditions in this area are satisfactory for oysters while D. marinum infections remain at a low level. The summer mortality that invariably occurs among older oysters maintained in Bayou Rigaud failed to develop in these young oysters, which had an extremely low incidence of the parasite. It has been repeatedly demonstrated that oysters developing heavy Dermocystidium infections likewise develop heavy mortalities. The above facts indicate that D. marinum is the important

mortality agent among oysters (protected from predators) in the Bayou Rigaud area.

It has been frequently observed that mortality is greater among older, market size oysters than among young oysters growing in the same area. In lower York River, Virginia, an area where D. marinum is prevalent, it was found that young oysters (under two years old) were more resistant to summer deaths than older oysters (Hewatt and Andrews, in press). McConnell (1936) reported an early fall mortality of large oysters in lower Plaquemines Parish, Louisiana, and he stated that in his opinion this mortality was due "to a high Mississippi River until late in the summer, succeeded by a very sudden drop requiring the oysters to acclimate themselves very quickly to water of high salinity in the hot summer time. The large oysters seemed to be unable to acclimate themselves as rapidly as did the younger ones and many died. There seemed to be, however, no unusual loss of the young oysters in the same beds."

The evidence obtained from the above studies suggests that the low degree of susceptibility of younger oysters to D. marinum may account for their better survival rather than the ability to adjust themselves to increasing salinities more rapidly than large oysters. The writer is not aware of any experimental evidence that suggests the greater adaptability of young oysters to such conditions. Furthermore, the fact that younger oysters show a very low incidence of infection as well as a low mortality in an area of high endemicity and high mortality

is additional epizootiological evidence that D. marinum is an important cause of mortality in such areas.

The data obtained from the above studies permit the following conclusions: (1) that oysters three to four months old are highly refractory to D. marinum infection; (2) that oysters from the third or fourth month to one year or longer are increasingly susceptible; (3) that oysters approximately a year or more old may become infected with D. marinum within three to four weeks when maintained in waters of high endemicity; and (4) that oysters setting in May or June show excellent growth and survive well (if protected from predators) in an area of high endemicity during the first year.

TABLE 5

Spat (Group A)

Date	No. Exam.	Approx. Age	Av. Lgth.[1]	% Pos.	Wghtd. Incid.	% Mortality Per Day
6-26-52 (Sta. 51)	26	1-2 wks.	10	0	0	---[2]
7-2-52 (S. H.)	25	2-3 wks.	14	0	0	---
7-17-52 (S. H.)	34	4-5 wks.	25	0	0	---
7-31-52 (Sta. 51)	27	6-7 wks.	34	0	0	0.08
8-22-52 (S. H.)	27	9-10 wks.	40	3.7	0.04	0.08
8-31-52	--	10-11 wks.	--	--	--	1.5[3]
9-19-52 (S. H.)	25	13-14 wks.	45	0	0	0.2[3]
10-11-52	--	16-17 wks.	--	--	--	0.25[3]
11-28-52 (S. H.)	15	23-24 wks.	55	6.6	0.07	0.27[3]
2-5-53 (S. H.)	15	33-34 wks.	56	6.6	0.13	0.1
4-3-53 (Sta. 51)	15	41-42 wks.	71	13.3	0.27	0.02
6-13-53[4]	54	51-52 wks.	81	37.0	0.66	0.14

[1] Average length, to nearest mm., of oysters in sample at time of culturing.

[2] Dashes indicate that no data were obtained.

[3] Mortality due mainly to small conchs.

[4] Sugar House Station (29 individuals) and Station 51 (25 individuals) combined.

TABLE 6.

Spat (Group B)

Date	No. Exam.	Approx. Age	Av. Lgth.[1]	% Pos.	Wghtd. Incid.	% Mortality Per Day
6-12-52	35	1-3 wks.	9	0	0	---[2]
6-26-52	25	3-5 wks.	17	0	0	---
7-18-52	26	6-8 wks.	31	3.8	0.08	---
8-30-52	--	12-14 wks.	--	--	--	0.33[3]
9-19-52	25	15-17 wks.	47	0	0	0.14[3]
10-11-52	--	18-20 wks.	--	--	--	0.19[3]
2-5-53	--	35-37 wks.	--	--	--	0.08
4-3-53	--	43-45 wks.	--	--	--	0.04
6-6-53	38	52-54 wks.	81	26.3	0.5	0.08

[1] Average length, to nearest mm., of oysters in sample at time of culturing.

[2] Dashes indicate that no data were obtained.

[3] Mortality due mainly to small conchs.

TABLE 7.

Oysters 11 to 12 Months Old.

Date	No. Exam.	Time in Bayou Rigaud	Per Cent Positive	Weighted Incidence
6-25-52	25	---[1]	0	0
7-2-52	10	1 wk.	0	0
7-9-52	10	2 wks.	0	0
7-16-52	10	3 wks.	0	0
7-18-52	10	3 wks. & 2 days	0	0
7-23-52	10	4 wks.	30	0.15
7-30-52	10	5 wks.	50	0.35
8-22-52	10	8 wks.	40	0.65
9-19-52	10	12 wks.	60	1.00
10-11-52	20	15 wks.	60	0.53

[1] Removed from lower Barataria Bay, Louisiana, about one month after setting and maintained at Bay Chene Fleur, Louisiana until June 25, 1952.

TABLE 8.

Long Island Oysters

Date	No. Exam.	Time in Bayou Rigaud	Per Cent Positive	Weighted Incidence	Per Cent Mortality Per Day
8-22-52	30	1 day	0	0	*
8-30-52	10	8 days	0	0	**
9-12-52	10	3 wks.	20	0.15	---[1]
9-19-52	10	4 wks.	30	0.25	---
9-26-52	10	5 wks.	20	0.10	---
10-3-52[2]	10	6 wks.	70	1.55	---
10-11-52	10	7 wks.	60	1.35	0.20
10-17-52	10	8 wks.	60	1.20	---
10-25-52	10	9 wks.	60	2.00	---
11-28-52	10	14 wks.	50	0.35	0.08
1-12-53	10	16 wks.	50	0.50	---
2-5-53	--	19.5 wks.	--	--	0.14
4-3-53	10	27.5 wks.	80	2.55	0.24
6-4-53	10	36.5 wks.	90	2.30	0.77

* Twenty-seven out of two bushels died within one day.

** Four died between 8-22-52 and 8-30-52.

[1] Dashes indicate that no data were obtained.

[2] A heavily infected "gaper" recovered on this date.

Figure 25. The incidence of Dermocystidium infection of three age groups of oysters maintained in Bayou Rigaud. Note: incidences of Group A spat (7-17-52) and Group B spat (7-18-52) combined and plotted at 7-18-52.

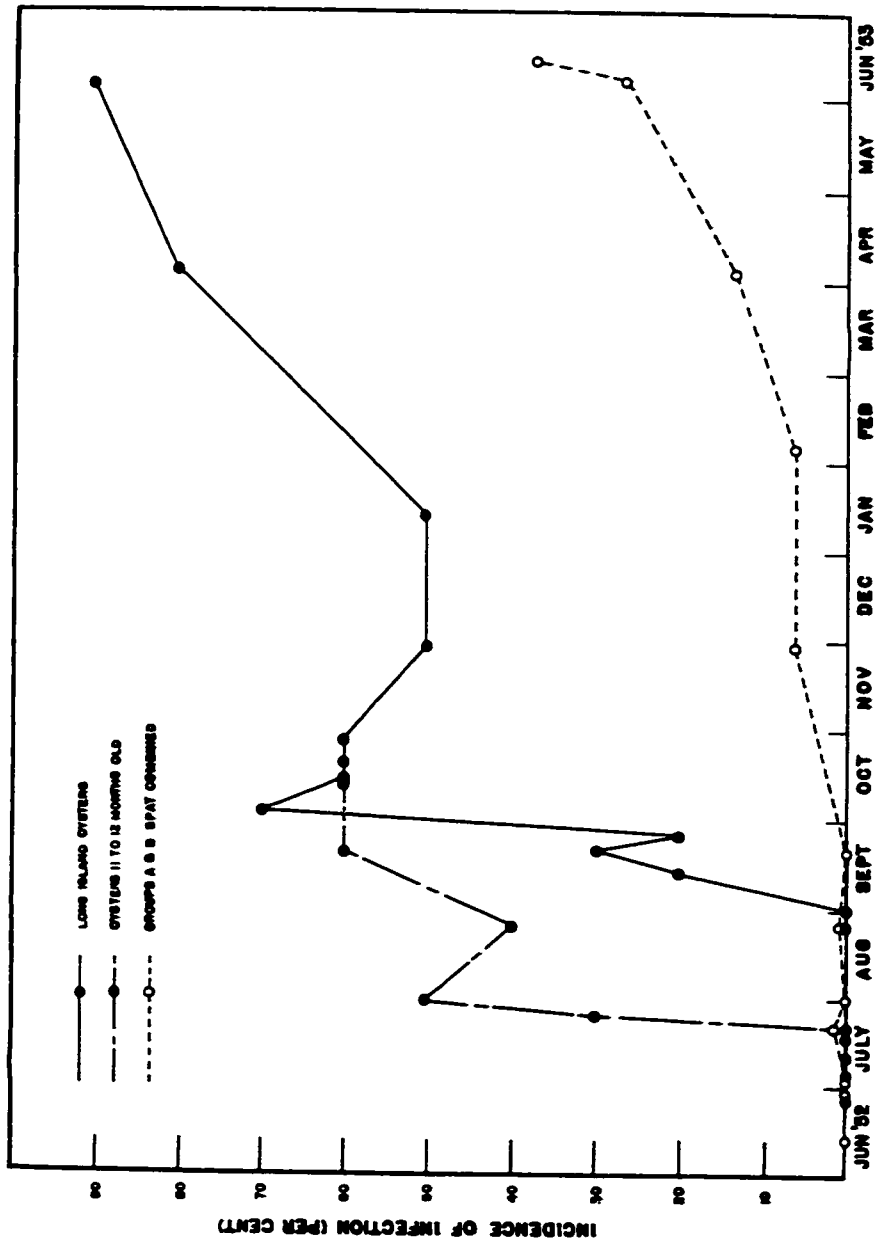

INCIDENCE OF INFECTION (PER CENT)
JUN '52
JULY
AUG
SEPT
OCT
NOV
DEC
JAN
FEB
MAR
APR
MAY
JUN '53

FIGURE 25.

Figure 26. The weighted incidence of Dermocystidium infection of the three age groups of oysters maintained in Bayou Rigaud. Note: weighted incidences of Group A spat (7-17-52) and Group B spat (7-18-52) combined and plotted at 7-18-52.

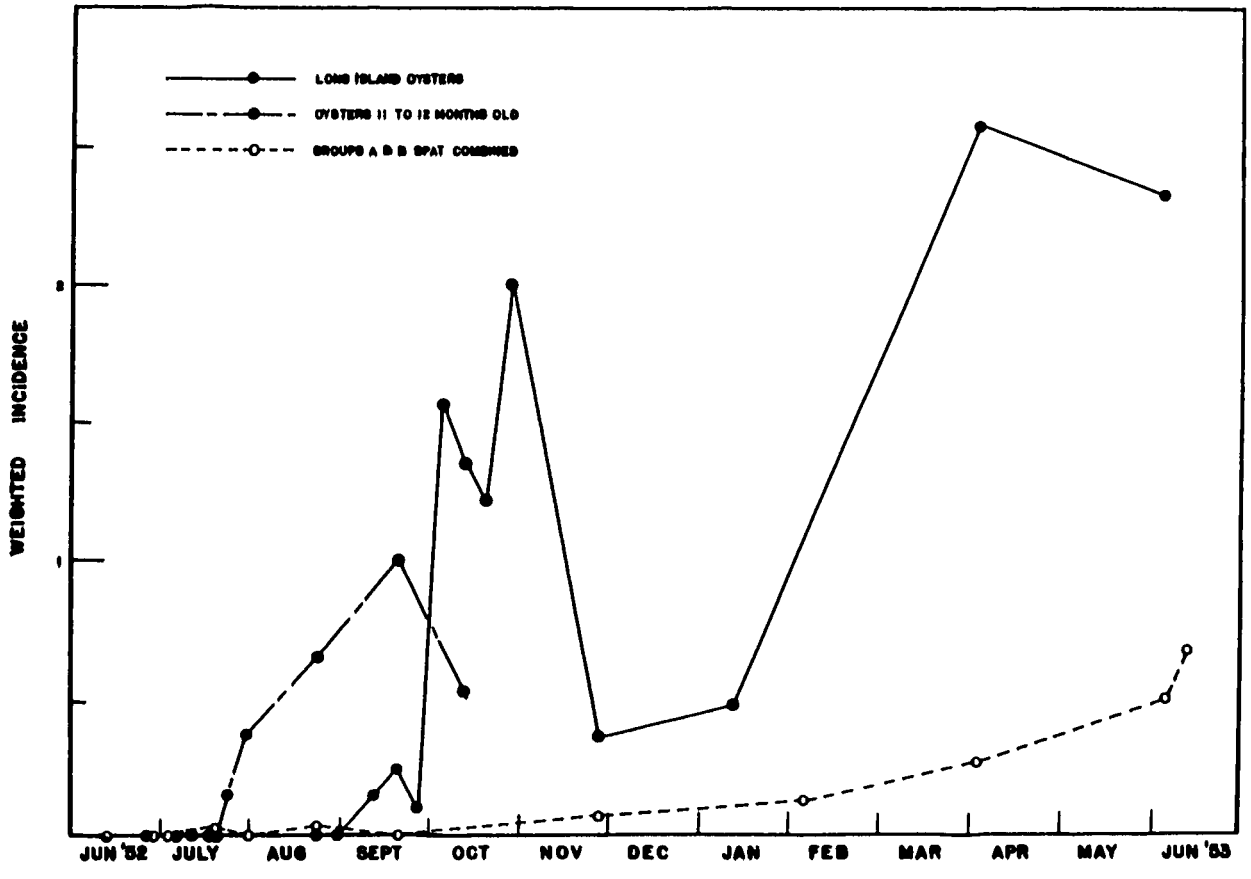

LONG ISLAND OYSTERS
OYSTERS 11 TO 12 MONTHS OLD
GROUPS A B B SPAT COMBINED
WEIGHTED INCIDENCE
2
1
JUN '52
JULY
AUG
SEPT
OCT
NOV
DEC
JAN
FEB
MAR
APR
MAY
JUN '53

FIGURE 26.

EPIZOOTIOLOGY AND DISTRIBUTION

1. Factors Controlling the Incidence and Intensity of D. Marinum.

a. Salinity.

As previously mentioned, high temperature and high salinity are intimately associated with high parasite incidence and high oyster mortality as well. For example, oysters show about 80 per cent mortality during the second summer and autumn when maintained in a high salinity area such as Bayou Rigaud. In order to have a constant supply of oysters infected to a high degree, therefore, it was necessary to remove periodically oysters (more than one year old) from the storage trays in Bay Chene Fleur---a low salinity area in which D. marinum occurs at a very low level except during extended dry periods. These transplanted oysters, which survive very well during the cooler months, develop high infection and high mortality during warmer months. The salinity generally ranges between 8 and 12/1000 at Bay Chene Fleur, whereas it seldom drops below 12/1000 at Bayou Rigaud; for the most part salinities range from 20 to 30/1000 during the warmer months. After extended dry periods, the salinity may exceed 20/1000 in Bay Chene Fleur; under these conditions there is an increase in incidence of the parasite and a slight increase in mortality. A return to normal salinity conditions results in a decrease in incidence. In Bay Chene Fleur the level of infection and mortality rises slightly in late summer and early fall but always remains far below the levels found in Bayou Rigaud. Mackin (unpublished data) found that mortality and Dermo-

cystidium infection were closely correlated at both Bayou Rigaud and Bay Chene Fleur.

The role of salinity in the epizootiology of Dermocystidium disease has been studied experimentally. It should be mentioned at this time that high salinity per se is not physiologically harmful to oysters; in colder waters oysters thrive well at high salinities where depredation from predators is not extensive. Gunter (1952) states that the American oyster (Crassostrea virginica) flourishes in waters with salinity approximately between 15 and 30/1000. Mackin (unpublished data) found that experimentally lowered salinities (about 11/1000) did not reduce or inhibit the development of the parasites in oysters previously infected. Mackin suggests that the correlation of D. marinum infections with salinity is not a result of physiological effects on either the host or the parasite, but is due to numerical dilution of infective elements by influx of fresh water and the subsequent flushing of the bay. This explanation assumes that D. marinum does not live in fresh water.

Experimental studies were conducted to determine the effect of low salinity on the infection rate of uninfected oysters. These experiments were conducted in closed aquaria and the water was changed weekly. The salinity of the water was adjusted to lower salinities by the addition of rain water. The "feeding" method of infection was used. The uninfected oysters were obtained from Redfish Bay, Louisiana, and ten of them were placed in each aquarium set up. The experiments

were begun on July 15, 1953. Water temperatures ranged from 24° to 31° C., exceeding 27° C. most of the time.

Two groups of experiments utilizing different amounts of infected tissue mince were performed simultaneously. In one group the oysters received five "feedings" of five mls. each at weekly intervals. In the high salinity aquarium the salinity varied from 26 to 28/1000 while it varied in the two low salinity aquaria from 10 to 13.5/1000.

The first acutely infected "gaper" appeared in the high salinity aquarium on the 37th day and the last oyster died on the 62nd day after the start of the experiment. All oysters except two that died within five days after the beginning of the experiment were heavily infected. In one of the low salinity aquaria the first acutely infected "gaper" appeared two days later than in the high salinity aquarium; in the other the first one did not appear until the 52nd day. In the first low salinity aquarium mentioned the last oyster died on the 73rd day and on the 90th day, October 13, 1953, in the other one. All of the oysters in both aquaria were heavily infected at time of death. When the last oyster died in the high salinity aquarium, there were three survivors in one low salinity aquarium and seven in the other. These results suggest that the rate of development of Dermocystidium infection was slightly delayed by the lower salinity; the final mortality, however, was equal in high salinity and low salinity.

In the second experiment the oysters received smaller dosages of infected material; the use of smaller dosages is probably more in

keeping with the conditions in nature. One low salinity (10 to 15/1000) and one high salinity (24 to 28.5/1000) aquarium were set up and each received five drops of infected tissue mince twice a week for about one month. The first acutely diseased "gaper" appeared in the high salinity aquarium on the 38th day---just one day later than was observed in the high salinity aquarium receiving the larger dosages. The last oyster died on the 76th day; all oysters but one, which died on the third day of experimentation, were heavily infected. The first acutely infected "gaper" appeared in the low salinity aquarium on the 64th day---26 days after the first one appeared in the high salinity aquarium. When the low salinity aquarium was closed out on October 13, 1953, 90 days after starting the experiment, one survivor remained. All oysters, including the lone survivor, were heavily infected except one that died about two weeks after the start of the experiment. At the time of the death of the last oyster in the high salinity aquarium there remained four live oysters in the low salinity aquarium.

These results, obtained with smaller dosages of infected material, indicate that the appearance of the acute stage of the disease is delayed nearly one month under the condition of low salinity. The final mortality, nevertheless, was the same under both conditions of salinity. Inasmuch as the infective elements probably accumulate in closed aquaria to an extent not found in nature, the effect of low salinity in retarding the development of lethal intensities of infection under natural conditions may be of more significance than the experimental data indicate.

In closed aquaria the host may not be able to cope with the large number of infective organisms present, whereas under natural conditions the oysters are probably subjected to fewer organisms at a time. Any factor which even slightly delays the development of infection may greatly enhance the oyster's ability to defend itself against infection.

Since the above studies demonstrate that low salinity is not physiologically unfavorable for the development of Dermocystidium infection, the exact role of salinity still remains obscure. The delay in development of Dermocystidium disease in low salinity in conjunction with the physical effects (flushing and dilution of more saline waters) as suggested by Mackin may account for the effect of salinity on the incidence and intensity of D. marinum.

b. Temperature.

Inasmuch as the epizootics produced by D. marinum display a seasonal periodicity (rising during warm months and declining during cool ones), it appears certain that temperature is the most important environmental factor affecting the incidence as well as the intensity of this parasite. In Bayou Rigaud, Mackin (unpublished data) found that fluctuations in incidence of the parasite are more sensitive to temperature variations than they are to salinity fluctuations. The incidence of infection rises sharply in areas of moderate to high salinities (exceeding 18/1000) when the water temperatures consistently exceed 20° C. The mortality and weighted incidence remain at a high level until the water temperatures drop below 25° C. The optimum development of

Dermocystidium infection occurs at temperatures ranging from 25° C. to the maximum summer temperatures, usually about 32° C. When temperatures consistently remain in the low twenties the mortality rate drops sharply; likewise the weighted incidence of the parasites in live oysters declines, although usually not as sharply as does the mortality rate. As previously mentioned, the host appears to be able to eliminate a large portion of the parasites during the winter and early spring months (see Figs. 25 and 26). Andrews and Hewatt (1953) working in Virginia found that infections were less frequent during the winter months and almost completely disappeared from February through May. In Virginia the mortality accelerates rapidly during July and reaches a peak during August and September, whereas in Louisiana (lower Barataria Bay and Bayou Rigaud) the mortalities usually increase rapidly during the late spring and early summer generally reaching a peak during July and August. The peaks, however, as well as the extent of the mortality periods, may be modified by the salinity effects pointed out above.

Mackin (1951b) found that oysters (over 50 per cent infected) in water cooled to 18° C. showed six times less mortality than a similar group of oysters receiving the same water but at natural temperatures (25° to 32° C.). Since the total incidence of infection was the same in both groups, it appears that the cooled water inhibited the metabolic activity of the parasites; but it did not cause an appreciable, if any, elimination of parasites as has been observed at still lower tempera-

tures (about 15° C. or less) in nature. In addition to reducing the metabolic activity of the parasites, the lower water temperatures probably greatly enhance the oyster's ability to combat this organism. This ability may be due to the fact that low temperatures favor continued pumping (Collier et al., 1953) with a resulting improvement in the condition of the oyster as indicated by increased glycogen deposits.

c. Other Factors.

Since the increase in mortality occurring in late spring is closely correlated with the usual peak spawning period, it has been frequently suggested that the weakening of oysters as a result of spawning may be responsible for the lethal effects of Dermocystidium infection. Mackin (1951b) found that only a very few oysters spawn when heavily infected; most "gapers" either failed to develop gonadal material or it was destroyed by the parasites. The lightly or moderately infected oysters developed normal gonads. He states, "It appears probable from these data that the correlation of spawning period and period of accelerating mortality is due to the fact that optimum temperature for Dermocystidium development corresponds very closely to the optimum spawning temperature, i.e., about 22° to 25° C. in Louisiana." The results of the pathogenicity experiments described above, conducted in the winter when spawning is not a factor, also indicate that the lethality of D. marinum is not due to weakness induced by spawning.. Furthermore, the method used to maintain a constant supply of highly infected oysters for experimental purposes during the winter

and early spring months also supplies additional evidence that temperature, not spawning weakness, is the primary factor controlling the development of Dermocystidium disease. Since in winter and early spring live oysters show a relatively low weighted incidence of the parasites, it was necessary periodically to bring live oysters into the laboratory and maintain them at approximately 25° C. to insure an adequate supply of highly infected oysters during the cool months. Under such conditions the weighted incidence rose rapidly and acutely infected oysters began to appear within three to five weeks. The evidence presented above clearly indicates that temperature is the most important environmental factor controlling the occurrence of D. marinum.

2. Distribution of D. marinum.

Natural infections with this parasite are now known to occur from the lower Chesapeake Bay region of Virginia to Rockport, Texas. The simplicity of the thioglycollate culture technique has greatly stimulated distributional studies of this fungus.

Mackin (1951a) found this organism in oysters from the James and Rappahannock Rivers, Virginia; Charleston, South Carolina; Pensacola, Florida; Gulfport, Mississippi; and Louisiana. Andrews and Hewatt (1953) reported that in Virginia the incidence of Dermocystidium was greatest in lower Chesapeake Bay, lower York River, and Hampton Roads (high salinity areas). They did not find it in the James River seed area (low salinity area). Andrews (unpublished data) has found a

single lightly infected oyster (possibly imported) from a group of 25 taken from lower Delaware Bay, New Jersey. Chipman (1954) finds D. marinum quite common in oysters around Beaufort, North Carolina. He states, "In all cases so far, however, the infestation would be rated as extremely light." During this investigation a small number of parasites were found in a few oysters from Wadmalaw River, South Carolina. There are no reports on the status of this organism in the state of Georgia. The writer does not believe that oysters from this state have been examined for Dermocystidium. Around Pensacola, Florida, where heavy summer mortalities frequently occur, the incidence is very high. The parasite has been reported from Apalachicola Bay and the Cedar Key area in Florida (Ingle and Dawson, 1953). Mackin (unpublished data) found a very high incidence of Dermocystidium at three stations in Mobile Bay, Alabama. The writer found a group of 30 oysters that had been maintained in experimental tanks at Biloxi, Mississippi, to be about 75 per cent infected.

By far the most extensive study of the distribution of this parasite has been conducted in the state of Louisiana. J. G. Mackin and the writer have checked oysters from about 125 stations located in this state, most of them being located west of the Mississippi River. D. marinum was found in varying abundance (incidence ranging from less than 5 per cent to 100 per cent) in all but seven of the stations. Two of the uninfected stations were located in Atchafalaya Bay, a low salinity area in the western part of the state, which receives a great amount of

fresh water from the Atchafalaya River. The five other stations were located adjacent to the Mississippi River Delta; four of these stations were in Redfish Bay, the other about eight miles offshore in Tiger Pass. In all of these areas the salinity was maintained at a low level by the river water.

This parasite has been found in oysters from several areas in middle and upper Galveston Bay, Texas. The incidence was fairly high in a large group of oysters taken from the intertidal zone in this bay at Seabrook, Texas. In spite of the extremely high salinities found in certain areas of Aransas Bay, Texas, D. marinum appears to be very uncommon in this south Texas area. Owen (unpublished data) found two oysters out of ten from the Rockport area to be infected. Mackin (unpublished data) failed to find it in two samples from Port Aransas. The writer found no parasites in a sample of 20 oysters from Tin Can Reef, Aransas Bay. The salinity was certainly high enough because the oysters were encrusted with a non-commercial oyster (Ostrea equestris) that thrives in high salinity waters. According to Gunter (1951), O. equestris lives in waters with salinities of 25 to 35/1000 and is not found far inside estuarine waters. The almost complete absence of Dermocystidium from the Rockport area is strange, for the environmental conditions (temperature and salinity) appear to be satisfactory unless extremely high salinities are unfavorable. In recent years the salinities in lower Aransas Bay have frequently exceeded that of pure sea water (35 to 36/1000). Hedgpeth

(1953) states, "The records of various years indicate that conditions have been extreme during the past several years, especially from 1948 through 1951, in Aransas Bay. Precipitation has been below average, and during 1950 the mean air temperature was higher than normal for every month. The result has been an increase in salinity, at times approaching 40/1000 in Aransas Bay at Rockport."

It is evident from the distribution records that D. marinum is very common and widely distributed along the South Atlantic and Gulf coasts of the United States. The writer is not aware of any records of its occurrence in other regions.

HOST-SPECIFICITY

1. Distribution of D. marinum or Related Parasites in Other Molluscan Hosts.

The possibility that other mollusks may serve as reservoir hosts for D. marinum was considered. To check this possibility a large number of mollusks of various species were examined for parasites behaving as D. marinum when cultured in thioglycollate medium. Among the other mollusks checked were three species of oysters in addition to the American oyster, Crassostrea virginica. A sample of 27 C. rizophorae (Guilding) from Puerto Rico, a commercial species known as the Cuban or mangrove oyster, was negative for parasites resembling D. marinum. Two out of 11 leafy oysters, Ostrea frons (Linnaeus), (identified by Dr. P. A. Butler) caught as spat and maintained in a tray for about two years in Santa Rosa Sound, Pensacola, Florida, were positive for organisms considered to be D. marinum.

On October 11, 1952, a large number of horse oysters, Ostrea equestris Say, were removed from Tin Can Reef, Aransas Bay, Texas, and placed in trays in Bayou Rigaud. The oysters were estimated to be over a year old. These oysters as well as the American oysters to which they were attached were free of Dermocystidium at this time. Both species of Ostrea are small, non-commercial forms found in warm, high salinity waters. A sample of 30 live O. equestris and one "gaper" examined on February 4, 1953, and one of 35 live ones checked on April 3, 1953, were negative for the parasite. One light infection was found

in a group of 35 live specimens examined June 2, 1953. In the meantime the incidence of infection in native C. virginica had reached high levels during the first part of April. About five weeks later (July 11, 1953) when the next sample of O. equestris was checked, the incidence of Dermocystidium showed a very sharp rise. The incidence of infection was 72 per cent (18 out of 25) with a weighted incidence of 2.1. Moreover, the mortality rate, which had been relatively low, increased rapidly at this time. This species of oyster appears to require considerably more time to become infected than the native commercial oyster; more than three months of warm weather was required for O. equestris to attain a high level of infection. Uzmann (unpublished data) was able to transmit D. marinum to the Olympic oyster, Ostrea lurida Carpenter, by exposure to infected Texas oysters. This oyster is a small commercial oyster found on the west coast of North America (Alaska to lower California).

In the lower York River region of Virginia, Andrews (unpublished data) has found several species of bivalves to be infected with parasites which enlarge in thioglycollate medium and stain blue when treated with iodine; with rare exceptions the mollusks were only lightly infected. A few species of mollusks were examined from Galveston Bay, Texas; Grand Isle, Louisiana; and Pensacola, Florida, as well as several species from Aransas Bay, Texas. As was found in the case of oysters from Aransas Bay, all of the other mollusks from that area were free of parasites resembling D. marinum. The forms found in most of the

other mollusks appeared to be similar to D. marinum, but a few showed some differences in cultural characteristics. These differences suggest that they are possibly not conspecific with the oyster parasite. Furthermore, cross-infection experiments to be discussed later suggest that some of them are different species. The mollusks examined and the results obtained as well as other pertinent data are presented below; they are listed in accordance with their scheme of classification (Pulley, 1952).

Class GASTROPODA

Order MESOGASTROPODA

Family CREPIDULIDAE

Crepidula plana Say (Flat Slipper)---This gastropod, usually found attached to oysters or their empty shells was not found infected under natural conditions. Ten (about six months old) from Pensacola, Florida, one from Grand Isle, Louisiana, and one from Aransas Bay, Texas, were examined. One lightly infected flat slipper was removed from the shell of a heavily infected oyster that had been in a closed aquarium with other infected oysters for nearly three months.

Crepidula fornicata Linnaeus (Slipper Limpet)---Found to be uninfected by Andrews in Virginia.

Order BASOMMATOPHORA

Family SIPHONARIIDAE

Siphonaria naufraga Stearns (= S. lineolata d'Orbigny) (Ship-wrecked

Siphon)---Seventeen from the jetties at Galveston, Texas, were negative.

Class PELECYPODA

Order TAXODONTA

Family ARCIDAE

Arca (=Anadara) transversa Say (Blood Clam or Transverse Ark)---A single individual from Aransas Bay was negative. This species was found infected by Andrews in Virginia.

Order ANISOMYARIA

Family MYTILIDAE

Brachidontes (=Mytilus) recurvus (Rafinesque) (Bent Mussel)---Thirty from Grand Isle and 12 from Galveston Bay were negative; all of these were removed from oysters that were 60 to 80 per cent infected.

Modiolus demissus Dillwyn (Ribbed Mussel)---Found to be uninfected by Andrews in Virginia.

Family PECTINIDAE

Pecten irradians (Lamarck) (Bay Scallop)---All individuals in a sample of 11 from Pensacola were infected with organisms appearing to be typical D. marinum. These scallops had a weighted incidence of 1.7 and the organisms were most abundant in the gill tissues and least abundant in the rectum; it may be recalled that the parasites are usually found most abundantly in the rectal tissues of the oyster. Chipman (1954) found this species lightly infected in the Beaufort, North Carolina, area.

Family ANOMIIDAE

Anomia simplex d'Orbigny (Plain Jingle)---Frequently found attached to other mollusks; was found to be infected with parasites resembling Dermocystidium. All of 11 individuals removed from the shells of Venus mercenaria from Virginia were infected. Two of six (about six months old) from Pensacola were infected; these had been maintained in an aquarium containing oysters for six weeks prior to being cultured. Nine individuals from Aransas Bay were negative.

Family OSTREIDAE

The species of oysters examined have been mentioned previously. The family to which they belong is inserted in this list in order to indicate the relationship of the oysters with the rest of the mollusks examined.

Order EULAMELLIBRANCHIATA

Family LUCINIDAE

Lucina pectinata Gmelin (=L. jamaicaensis Lamarck) (Jamaica Lucina) ---Twenty-six specimens from Aransas Bay were negative.

Family CARDIIDAE

Dinocardium robustum (Solander) (Strong Cockle)---Five of these cockles that had been washed up on the beach at Port Aransas, Texas, were negative.

Laevicardium mortoni Conrad (Morton's Cockle)---One from Aransas Bay was negative. Andrews found this species infected in Virginia.

Trachycardium muricatum (Linnaeus) (Common Cockle)---One specimen from Aransas Bay was negative.

Family VENERIDAE

Chione cancellata Linnaeus (Cross-barred Venus)---Four individuals from Aransas Bay were negative.

Venus campechiensis Gmelin (Hard Shell Clam)---One from Quarantine Bay, Louisiana, and one from Aransas Bay were negative.

Venus mercenaria Linnaeus (Hard Shell Clam)---Found infected by Andrews. A number of lightly infected clams received from Virginia were maintained in closed aquaria at approximately 25° C. for two months. Under such conditions no increase in intensity of infection was observed in the clams; lightly infected oysters, however, generally show a marked increase in intensity when so treated. Attempts to infect this species of clam under natural conditions have thus far been unsuccessful. On August 10, 1953, a large number of uninfected Venus mercenaria from Milford, Connecticut, were placed in Bayou Rigaud. Eight live clams examined on October 19, 1953, were free of Dermocystidium. A large number of "gapers" also proved to be negative. The number of "gapers" and the month in which they were recovered were as follows: 2 during the last two days of August; 21 during September (13 recovered after September 20); 6 during October; 1 during November; and 1 during December. These results indicate that Venus mercenaria, if susceptible

tible to D. marinum, requires a much longer time to become infected than oysters. As previously pointed out, oysters from Milford, Connecticut, became infected after one to three weeks in Bayou Rigaud. Infection experiments to be discussed shortly demonstrate that the hard shell clam is a poor host for the oyster parasite.

Family MACTRIDAE

Mactra fragilis Gmelin (Frail Clam)---Twenty-three individuals from Aransas Bay were negative.

Mulinia lateralis (Say) (Little Surf Clam)---Thirty-two specimens from Aransas Bay were negative. Andrews found this species to be infected in Virginia. It was one of the few species that showed greater than light infections.

Family DONACIDAE

Donax variabilis Say (Veriable Wedge)---Forty-three individuals from Galveston Beach, Galveston, Texas, were negative.

Family SANGUINOLARIIDAE

Tagelus gibbus (Spengler) (Stubby Razor Clam)---Sixteen specimens from Aransas Bay were negative. This species was found infected by Andrews in Virginia.

Family TELLINIDAE

Tellina tampaensis Conrad (Tampa Tellin)---Thirty individuals from Aransas Bay were negative.

Macoma spp. ---Andrews found infected individuals in three species of

this genus. All specimens of M. balthica (Linnaeus), of which about 100 were checked either by Andrews or the writer, were infected. The majority were lightly infected; a few moderate infections were observed. Evidence to be presented later indicates that the organism found in M. balthica is not the same species as D. marinum. The other two species found infected are M. tenta (Say) and M. phenax (?).

Family SOLENIDAE

Ensis minor Dall (Razor Clam)---Eleven specimens from Aransas Bay were negative. This species was found infected by Andrews in Virginia.

Family MYACIDAE

Mya arenaria Linnaeus (Soft Clam)---Found infected by Andrews in Virginia.

Family PHOLADIDAE

Martesia sp. (Boring Clam)---Commensal in the shells of oysters. Two of seven clams removed from the shells of oysters (about 75 per cent infected with D. marinum) from Biloxi, Mississippi, were infected with organisms appearing to be somewhat similar to the oyster parasite. This organism, however, occurred only in tight clusters, some of which appeared to be surrounded by a thin membrane. They were smaller in size than the typical oyster parasite and they appeared to occur only in the mantle tissue. It is not considered that these organisms were the same as those

found in oysters. These parasites were not encountered in ten boring clams from Grand Isle. A single clam removed from a heavily infected oyster maintained in a closed aquarium for three weeks, was found to contain 10 to 15 organisms appearing to be typical D. marinum.

Family LYONSIIDAE

Lyonsia hyalina (Conrad) (Glassy Lyonsia)---Andrews found this species to be infected in Virginia.

Family PERIPLOMATIDAE

Periploma inaequivalvis Schumacher (Inequal Spoon)---One specimen from Aransas Bay was negative.

It is obvious from the above list that parasites, which enlarge in thioglycollate medium and stain blue with iodine after such enlargement, are widely distributed among pelecypod mollusks. Thus far 18 species, comprising some 13 families and belonging to three orders, have been found to harbor Dermocystidium or related organisms. When more mollusks are checked from the endemic areas, this list will probably be extended. A comparison of the results obtained with similar species (a total of six) examined from lower York River, Virginia, and Aransas Bay, Texas, suggests that an area free of D. marinum in oysters is also free of the same or related species in other mollusks. Unfortunately it was possible to obtain only a relatively few species of mollusks from three areas (Pensacola, Barataria Bay, and Galveston Bay) of high endemicity for D. marinum.

2. Attempts to Transfer D. marinum or Related Parasites Experimentally from One Host to Another.

After the discovery by Andrews that other mollusks were infected with Dermocystidium-like parasites, experimental studies were conducted to determine the degree of host-specificity of two of the parasites. Data obtained from such studies were expected to give some indication as to whether or not the parasites from the different hosts are conspecific. Cross-infection experiments were attempted using the parasites from southern oysters and from Macoma balthica from Virginia. Plans to use the parasites from Venus mercenaria from Virginia were abandoned since these clams were too lightly infected to be used. The uninfected hosts used were oysters, hard shell clams (Venus mercenaria), and soft shell clams (Mya arenaria) from Milford, Connecticut. All experiments were conducted in closed aquaria and the water was changed once a week. Water temperatures averaged about 25° C. most of the time.

Attempts at cross-infecting the parasites from Macoma balthica will be discussed first. On November 6, 1953, 17 uninfected Mya were "Injected" through the mantle with 1/2 ml. of tissue mince prepared from lightly to moderately infected Macoma (12 minced in about 50 mls. of sea water). The "injected" clams survived very poorly and the last one died on the 20th day after the start of the study. Although a very few organisms were found in the gill tissues of four of the early "gapers", there was no evidence of invasion and multiplication of the parasite.

Twelve clams received a single "feeding" of infected Macoma mince. The last one died on the 45th day; not a single organism was observed in the dead clams.

Seventeen uninfected Venus were "injected" with one ml. of infected Macoma mince on November 6 and 19, 1953, and seven Macoma "gapers" were placed in the aquarium at various times. The experiment was discontinued after 113 days; there were four survivors. A few of the "gapers" appearing within one month after initiation of the experiment showed a very few organisms (only rare ones in the tissue) in or on the gill and mantle tissues; there was no evidence of invasion and multiplication of the parasites. Six clams were also placed in the aquarium that received 15 mls. of Macoma mince. One clam survived when the aquarium was closed out 113 days later. The five dead clams and the survivor were free of parasites.

Seventeen uninfected oysters "injected" with one ml. of infected mince on November 6 and 19, 1953, and six Macoma "gapers" were placed in the aquarium at various times. The first oyster died one month after the start of the experiment and the last one died after two months had elapsed. Not a single parasite was observed in any of the four tissues from the 17 oysters. Six oysters were placed in the aquarium receiving 15 mls. of infected mince; two oysters survived when the study was discontinued after 113 days. All six of the oysters were uninfected. Since no uninfected Macoma balthica were used as controls, it is possible that the concentration of the parasites in the mince was

was not sufficient to cause infection. It is, however, the writer's opinion that the inoculum was adequate to infect the three hosts used. The results suggest possibly that the oyster, hard shell clam, and soft shell clam are not suitable hosts for the Dermocystidium-like parasites found in Macoma balthica.

The two species of clams and the oyster were "injected" with heavily infected oyster tissue mince. Seventeen Mya were "injected" with 1/2 ml. of the mince. These clams survived somewhat better than those that received the Macoma mince. Six of the clams died between the fifth and tenth days---two were uninfected, once lightly infected, and three moderately infected. The rest of the clams died between the 12th and 42nd days, six surviving a month or more. Four of them were negative and the other seven showed very few (usually less than ten) organisms in the gill and mantle tissues. With the exception of the three moderately infected individuals which died within ten days none of the clams showed appreciable invasion and proliferation of the parasites. Two possible explanations for the three moderate infections are: (1) since clams that died during the early days were probably in a weakened condition, they were unable to resist the invasion and subsequent proliferation of the parasites; (2) since a heavy inoculum was used, the parasites may have invaded and proliferated in the tissues of most of the clams and only those that survived longer than ten days were in suitable condition to destroy the majority of the parasites.

Twelve Mya were placed in an aquarium which received four "feedings" of five mls. each of heavily infected oyster mince at weekly intervals. All but two died within two weeks; two survived for nearly two months. All proved to be free of the organism. The above results suggest that the soft clam is a poor host for the oyster parasite.

Seventeen of the hard shell clams were "injected" with one ml. of the same infected oyster mince as used above. Since the edge of the mantle is attached near the margin of the shell, it was necessary to drill through the mantle on one side in order to "inject" these clams. The first death occurred on the 19th day and 11 survived from one to four and one-half months. There were two survivors when the experiment was discontinued after four and one-half months. After examination of the first six "gapers", it became evident that most of the invasion and multiplication of the parasites occurred in a more or less localized area near the ventral margin of the mantle. This limited distribution suggested that the parasites gained entrance into the mantle tissue at the site damaged by drilling. All subsequent "gapers" and the two survivors were cultured in the following manner. The major portion of the mantle and gill tissue from the "injected" side along with the heart, rectum, and a piece of the digestive gland were cultured in a single tube while the gill and mantle tissues from the "uninjected" were placed in another tube. The gill and mantle tissues were examined without teasing so that the location of the parasites could be observed. Three of the clams were completely free of parasites. With the excep-

tion of a rare, abnormal-appearing parasite near the surface of the tissue, no parasites were observed in the tissues from the "uninjected" side, whereas the tissues from the "injected" side, especially mantle tissue, showed concentrations ranging from a very few scattered organisms to fairly large clusters in a more or less localized area near the ventral margin of the mantle. The gill tissue contained only a few parasites. Some of the infections in the mantle were estimated as moderate in the localized area. The major portion of these tissues, however, was free of parasites. There was no evidence that such organs as the heart, rectum, or digestive gland were invaded. Such results support the suggestion that the damaged area of the mantle provided the main portal of entry for the parasites. In one of the survivors the parasites appeared to have multiplied within the mantle cavity without extensive invasion of the gill and mantle tissues. When the tissues were examined a large number of organisms were found attached to the surface of the tissues and only a relatively few were found within. Six Venus placed in the aquarium that received the four "feedings" of infected oyster mince survived for nearly five months and all were found to be uninfected. Since large inocula of heavily infected mince failed to produce extensive infections beyond the site of "injection", it appears certain that Venus mercenaria is a very poor host for D. marinum.

The uninfected control oysters which were "injected" with the same mince as used to "inject" the Mya and Venus became infected

readily. Six of the oysters died within 18 days after "injection"; all were infected although the infections were not acute. This early mortality was attributed mainly to excessive fouling of the water by decomposition of the boring sponge infesting the oysters. The first acutely infected "gaper" appeared on the 20th day. Between the 20th and 53rd days the remaining ten oysters died and all were heavily infected. The six oysters that were placed in the aquarium with the Mya and Venus and received the four "feedings" of oyster mince became heavily infected. The first one died after 74 days and the last one died after 137 days. These results clearly indicate that failure of the clams to become infected to an appreciable degree was not due to insufficient concentration of infective elements. Furthermore, the writer believes that sufficient time elapsed before death of all of the hosts or discontinuation of the studies for some infections to have taken place.

During this study it was observed that in culture the oyster parasite enlarged more slowly in Venus than in the oyster; about one week was required for the parasites in the clam tissue to attain the size usually reached by the parasites in the oyster tissue after 48 to 72 hours. This reduced rate of enlargement was also observed in the naturally infected clams from Virginia.

The failure to successfully cross-infect oysters, soft shell clams, and hard shell clams with the Macoma parasite and the similar failure to transmit the oyster parasite to these two clams indicate

a fairly rigid host-specificity. The results also suggest that the two parasites are not the same, at least physiologically; they also suggest that the organisms found in Venus mercenaria and Mya arenaria by Andrews in Virginia may be different from either the Macoma or the oyster parasite. Future investigations will probably reveal a large complex of fungous organisms that respond to the thioglycollate culture technique in a similar manner.

DISCUSSION OF SIGNIFICANCE TO OYSTER INDUSTRY

It has been discovered that a large part of the heavy summer mortality of oysters on the Gulf coast is caused by D. marinum, a fungous parasite of proven pathogenicity for oysters. The studies reported herein were greatly facilitated by the rapid diagnosis of incidence and intensity of infection made possible by the discovery of the thioglycollate culture technique. The analysis of ecological and experimental data revealed that Dermocystidium infection is favored by high temperature (above 20° C.) and high salinity (above 18/1000).

The impact of Dermocystidium on the industry is recognized by oyster biologists. There remains the problem of developing practical industrial techniques to mitigate the ravages of this parasite. Before proceeding with the discussion of possible solutions to this problem, it is desirable to describe briefly the technique of oyster culture. The description will be based on the methods practiced in Louisiana since this is the only Gulf state in which there is significant oyster cultivation.

The seed oysters (small oysters) may be obtained by planting clean shells in a suitable area in late spring or early summer so that the larvae may "set" on them. These young oysters may be left where they are caught or they may be transplanted to other areas. The most common practice is to remove seed oysters (usually a year old or less) from the crowded natural reefs, which are located mainly east of the Mississippi River in Louisiana. The seed grounds (either natural reefs

or areas planted with shells by the state) are usually located in areas of relatively low salinity. Such areas are conducive to the production of tremendous numbers of small oysters; furthermore, the reduced salinity is unfavorable for predators which are very destructive to young oysters. The oysters on the seed grounds are usually too crowded to grow well; when transplanted to more saline waters where they are scattered, these oysters subsequently grow and fatten rapidly into counter stock (those served on the half-shell). In Louisiana the seed oysters are usually planted very thickly on the cultivated beds; oystermen report (Galtsoff et al., 1935) that 1,500 to 2,000 bushels are planted per acre. Since it has been shown that D. marinum may be transmitted from oyster to oyster, the overcrowding of oysters on the cultivated beds probably accelerates the spread of the parasites within transplanted populations. In areas where Dermocystidium is prevalent, the effect of overcrowding on the parasite incidence and mortality should be investigated. It is possible that a reduction in the concentration of planted seed oysters may reduce the mortality rate.

According to McConnell (1950), the oystermen in Louisiana have had to modify their practices within the last 10 to 15 years. Formerly, small seed oysters were transplanted to beds west of the Mississippi River during February, March, and April; these oysters were usually harvested about 18 months later. This procedure required that the oysters remain on the cultivated beds for two summers. In recent years, however, excessive mortalities occurring during the second

summer have resulted in extensive losses of large (counter) oysters. Later the planters found that they could not leave them on the beds for even one summer without sustaining heavy losses. At the present time therefore, to avoid the summer losses, the largest seed oysters available are planted from August 15 to December 1 of each year and they are harvested before the summer. This present practice, however, places an excessive drain on the seed grounds since the oystermen use 6 to 8 times as many seed as they formerly did (McConnell, 1950). Unless the seed oysters are fairly large to begin with, oysters harvested after this short period are usually too small for counter stock; such oysters are used largely for canning.

The oystermen appear to have found a practical method for avoiding these summer losses without knowing the causes of them. It may be recalled that uninfected northern oysters transplanted to Bayou Rigaud in late August suffered little mortality until the following April and June; those planted in early June, however, showed about 80 per cent mortality by the end of October. In the first instance, the oysters developed a fairly high Dermocystidium infection after six weeks in waters of high endemicity. As a result of lower water temperatures prevailing in late autumn, however, the intensity of infection dropped and both the intensity of infection and mortality remained at a relatively low level until April of the following year. On the other hand, those oysters transplanted during early June were subjected to high temperatures for about four months, and as a result about 80 per cent of them

died of Dermocystidium infection. The results obtained in actual practice as well as those obtained experimentally demonstrated that oysters which are susceptible to D. marinum (those approximately one year or more old) should remain in high salinity waters only a relatively short time during the warmer months.

In considering localized areas the main factor associated with the progressive development of warm weather mortalities appears to be the increasing saltiness of the water. Excessive saltiness of the water during the warmer months has been blamed either directly or indirectly for these mortalities by a number of oystermen and most biologists. McConnell (1942) in commenting on the high mortalities among the most highly cultivated counter oysters in Plaquemines and Jefferson Parishes, Louisiana, during August and September, states, "Because for the past several years now the annual flow of the Mississippi River has been considerably under normal, the finger of suspicion points to a lack of fresh water as either the direct or indirect cause of losses noted."

Frequent requests have been made by oystermen and others for the development of controlled means of diverting Mississippi River water to the more saline oyster growing areas west of this river at the times when excessivly high salinities prevail. Such diversions would have to be carefully controlled since prolonged exposure to very low salinities (below 5/1000) will kill oysters (Butler, 1949). The encroachment of highly saline waters into the bays and inlets has pro-

gressed steadily in recent years with the result that the more inland areas have become suited for oyster production. Gunter (1952) gave an excellent review of the historical changes in the Mississippi River and the adjacent marine environment. He pointed out that changes in the river flow, erosion of barrier islands, widening of passes to bays, rising of the sea level, and sinking of the Louisiana coast are responsible for the steady encroachment of waters of high salinity into the Louisiana bays in the vicinity of the Mississippi River. Owen (1953a) compared the oyster production (1932-1950) of five parishes (St. Bernard, Plaquemines, Jefferson, LaFouche, and Terrebonne) of southeast Louisiana with the climatological factors of temperature and rainfall. He found that when the air temperature was 27.8° C. or over and the rainfall was 7.0 cm. or under for prolonged periods during the preceding summer, the oyster production decreased significantly during the following season. On the other hand, he found that production increased if preceded by a relatively cool-wet summer. He concluded that mortality rather than failure to grow was responsible for the decreased production.

The direct correlation of Dermocystidium infection as well as oyster mortality with high salinity suggests that the chief effect of high salinity is the enhancement of the development of D. marinum infections. The role of high salinity in favoring the occurrence of oyster predators (especially Thais haemostoma), which are particularly destructive to young oysters, is well known and needs no further comment.

Another fact supporting the above suggestion is that young oysters (protected from predators), which had a low level of infection with this parasite for nearly a year, survived and grew well in a high salinity area. In addition, experimental evidence indicates that low salinity retards the development of acute infections in oysters previously uninfected. It seems certain from all of the above evidence that excessive warm weather mortalities of older oysters, appearing to be due largely to Dermocystidium disease, can best be prevented either by growing oysters in waters of relatively low salinity or by controlled diversion of fresh water to the high salinity areas during the warmer months, thereby reducing the salinity. This evidence also confirms the validity of the oystermen's request for the controlled diversion of fresh water from the Mississippi River mentioned above.

The supply of fairly large counter oysters may possibly be increased by taking advantage of the fact that young oysters are only slightly susceptible to D. marinum infections. The procedure would call for the planting of the youngest seed oysters possible (preferably those caught as spat in late summer or early fall) during late autumn, at which time the activity of the conchs is greatly reduced. The oysters would pass through the first summer as they approached one year of age. As previously pointed out, very young oysters (three to four months old) are highly resistant to Dermocystidium, even under conditions most conducive to optimum development of infection. This resistance to infection was found to decrease more or less gradually until,

approximately one year after "setting", an incidence of about 33 per cent and a weighted incidence of 0.6 were attained. The mortality was relatively low at this time. In view of such evidence it is believed that most of the oysters of this age (approaching one year) would survive until the following autumn if the damage by conchs were not too great. During late autumn the damage from both Dermocystidium and conchs would be greatly reduced by the lower water temperatures. If these oysters were harvested either late in the following winter or early in the spring, they would have remained in the more saline waters for 15 to 18 months, thus attaining the large size as well as excellent flavor required for the counter trade. An examination of Fig. 26 shows that the weighted incidence of D. marinum in oysters 11 to 12 months old did not exceed 1.0 although they were maintained in Bayou Rigaud for nearly four months (June 25 to October 11). It has been consistently observed that mortalities from Dermocystidium remain at a low level when the weighted incidence does not exceed 1.0. Although no mortality records were obtained for the 11 to 12 month old oysters mentioned above, very few deaths were observed among them.

Another method of possibly increasing the supply of large counter oysters, if the costs were not excessive, would consist of transplanting the susceptible oysters (those a year or more old) from high salinity to low salinity areas during the early spring, allowing them to remain in such areas until late August. These oysters could then be returned to high salinity areas where the size, shape, and flavor qualities de-

sired for counter oysters might be attained. The costs of the extra transplantings might possibly militate against this procedure.

The problem of reducing the oyster mortalities may also be approached by attempting to develop or select seed oysters that are more or less resistant to D. marinum infection. Hewatt and Andrews (in press) found that oysters (one month old when obtained) from South Carolina showed continued resistance to summer mortality factors through the second year in an area of high endemicity in Virginia. A more resistant stock may possibly be developed by selecting as breeding stock the survivors of either natural or experimentally induced epizootics. The observations of Hewatt and Andrews mentioned above suggest that attempts to develop or select a more or less resistant strain may lead to a possible reduction of the damages from D. marinum. Of course it would be very difficult to prevent the dilution of a resistant stock in nature should one be developed or selected. Should resistant stocks be developed it would be necessary to propagate them in areas more or less isolated to prevent or greatly reduce dilution by non-resistant oysters. When these resistant seed oysters attained a suitable size, they could be transplanted to the cultivated beds.

The development of a strain of oysters resistant to a disease is not without precedent. For example, the oysters of Prince Edward Island, Canada, (southern part of the Gulf of St. Lawrence) were practically wiped out by the progressive spread of a contagious disease of unknown etiology (Needler and Logie, 1947). The mortalities were of

the same magnitude and showed a similar seasonal periodicity (peak mortality during late summer and autumn) as those occurring on the cultivated beds in Louisiana. A small population of survivors remained in Malpeque Bay; the resistance of this population gradually increased over a period of 13 years until the population became almost completely resistant. These oysters were used to restock other bays where the native populations were surviving poorly. In all cases where these resistant oysters were introduced the production gradually increased and the industry was re-established. Since heavy mortalities developed within a year among oysters from other areas when introduced into Malpeque Bay, it was evident that the disease causing agent was still present.

The question may be raised as to why resistant populations similar to those mentioned above have not developed in areas of high Dermocystidium endemicity. There are several possible answers to this question: (1) resistant populations may have developed and as yet have remained undetected; (2) the practice of removing for market the largest oysters from a population may selectively remove those oysters most likely to produce more or less resistant offspring; and (3) most small oysters developing as a result of a natural "set" in an area of high endemicity are probably destroyed by predators. Since there are areas of endemicity in moderately saline waters where predators are less prevalent, it is possible that a resistant population may gradually develop in nature if some of the larger oysters are allowed to remain on the beds.

SUMMARY AND CONCLUSIONS

1. A protistan parasite, Dermocystidium marinum, was recently discovered and suspected as causing extensive mortalities of its host, Crassostrea virginica. D. marinum is the only parasite referred to this genus to be reported from estuarine environments. Its life cycle is unknown, but it is definitely a fungus and resembles yeast-like mycotic disease-producing organisms such as Cryptococcus and Blastomyces.

2. Annual oyster mortalities confined to the warm months have been recurring both on the Gulf and South Atlantic coasts and, until recently, have been little understood. Evidence has been brought forth that a large part of this mortality is associated with D. marinum infection. It was the objective of this investigation to produce information regarding the transmission and pathogenicity of this parasite.

3. The diagnosis of the parasite is accomplished by culturing various oyster tissues in tubes of thioglycollate medium for a minimum of 48 hours at room temperature and subsequently staining the tissues with iodine solution. A positive diagnosis is indicated by the presence of blue, sphrical, cystlike bodies, usually reaching a maximum diameter of 70 to 125 μ. Although hyphal and budding forms have been observed, appreciable multiplication does not occur.

4. The nutritional and environmental factors required for the enlargement and the germination of D. marinum were studied. Yeast ex-

tract and dextrose proved to be the essential components of thioglycollate medium. The production of hyphal forms was observed most frequently, although not consistently, in medium containing yeast extract and dextrose and appeared to occur more frequently during cooler months. Attempts to increase the production of hyphal forms by variation of incubation temperatures as well as nutritional components and salinity of the medium proved unsuccessful. Various solid media failed to support growth. Enlargement occurred at temperatures ranging between 10° and 30° C. and at salinities ranging between 3.5 and 50/1000. Thioglycollate medium rehydrated with distilled water containing 20 grams NaCl per liter resulted in the most uniform enlargement of the parasites. Small amounts of copper stimulated growth while large amounts caused death.

5. Four methods of experimentally transmitting D. marinum were investigated, namely: (1) "proximity", in which uninfected and infected oysters were placed in the same aquarium; (2) "feeding", in which infected tissue mince was placed in aquaria containing uninfected oysters; (3) "injection", in which infected tissue was "injected" into the mantle cavity of uninfected oysters; and (4) in vitro, in which small pieces of living oyster tissues maintained in sterile sea water were subjected to small quantities of infected pericardial fluid. All methods of transmission proved to be successful. During warm months acute infections developed between two and three weeks after "injection" and about one month was required by the "feeding"

method. Direct transmission was demonstrated and it constitutes the only proven method of transmission.

6. The pathogenicity of D. marinum was demonstrated conclusively since the death rate among experimentally infected oysters was more than twice as great as that occurring among similarly treated uninfected controls. In five of eight studies, the experimental oysters had a mortality rate 6.5 to 13 times greater than the controls. Only those experimental oysters dying during the early days of the studies, presumably from other causes, failed to develop acute infections. The rate of the experimental development of acute infection as well as mortality was similar to that occurring under natural conditions in an area of high endemicity.

7. An important pathological effect of parasitization was an appreciable loss of weight, resulting chiefly from the lysis of the host tissues. The relative weight loss was directly correlated with the intensity of infection. Those moderately infected lost about 12 to 15 per cent of their normal weight while heavily infected ones lost from 20 per cent (in cool months) to 50 per cent (in summer).

8. It was found that a significant age differential existed in the degree of susceptibility to infection. Oysters three to four months old are highly refractory to infection; from this age to one year or older the susceptibility increases more or less gradually. Southern oysters about one year old became infected after about four weeks in waters of high endemicity while northern oysters three to four years

old placed in the same waters became infected after one to three weeks. Oysters collected and maintained in an area of high endemicity showed excellent growth (well shaped and deeply cupped) and survival (when protected from predators) during the first year, attaining an average length slightly greater than the minimum marketable size of three inches. At this time these oysters showed an incidence of infection of 33 per cent and a weighted incidence of 0.6.

9. Temperature and salinity appear to be the important environmental factors controlling the incidence and intensity of the parasite. In Louisiana the incidence of infection and oyster mortality rises sharply in waters exceeding 18/1000 salinity when temperatures consistently exceed 20° C. The optimum development of D. marinum and greatest oyster mortality occur between 25 and 32° C. During colder months (15° C. or less) oysters are able to rid themselves of most of the parasites. Low salinity (10 to 15/1000) was found to retard the development of acute infection experimentally.

10. D. marinum is known to occur from lower Chesapeake Bay region in Virginia to Rockport, Texas. Its almost complete absence from Aransas Bay, Texas, where salinities often exceed that of pure sea water, suggests that excessively high salinities are unfavorable for the parasite.

11. D. marinum or related parasites were found widely distributed in other mollusks, especially in Virginia. Three species of oysters of

the genus Ostrea also serve as hosts. Eighteen species of pelecypod mollusks comprising some 13 families and belonging to three orders are known to harbor parasites detectable by the thioglycollate technique. The results of cross-infection attempts indicate that some of the forms are not conspecific and also that a fairly rigid host-specificity exists. The parasite of Macoma balthica did not develop when "injected" into oysters, hard shell clams, and soft shell clams. The oyster parasite failed to produce appreciable infections when "injected" into the two clams mentioned above. The hard shell clam remained refractory to D. marinum after extended exposure in an area of high endemicity.

12. The experimentally proven pathogenicity of D. marinum in conjunction with the extensive epizootiological evidence associating it with oyster mortality unquestionably establishes the economic importance of this parasite. Some possible remedial measures to reduce the losses incurred from this parasite were suggested. These measures include (1) planting of very young seed oysters in late autumn and harvesting prior to the second summer, (2) transplanting of oysters from high salinity to low salinity just prior to the warm seasons and returning them to the more saline areas at the beginning of the cool seasons, and (3) attempting to develop or select a resistant strain of seed oysters.

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