Table 1. Effect of various treatments on the Feulgen staining of symbiotes and nuclei of follicle cells.*

1st treatment	2nd treatment	Feulgen reaction	
		Follicle nuclei	Symbiotes
None	DNAse	−	+
	DNAse control	+	+
1*N* HCl at 60°C; 10 min	DNAse	−	+
	DNAse control	+	+
RNAse; 0.2 mg/ml; 56°C for 2 hr	DNAse	−	+
	DNAse control	+	+
	None	+	+
RNAse control	None	+	+
Saliva; 30 min; room temp.	DNAse	−	+
	DNAse control	+	+
2X crystalline pepsin; 2.0 mg/ml in 0.02*N* HCl; 37°C; 45 min	DNAse	−	−
	DNAse control	+	+
	None	+	+
Pepsin controls	None	+	+

* DNAse: 0.1 mg/ml in phosphate buffer *p*H 7.5 with gelatin and magnesium sulfate at room temperature for 45 min.
Controls: same conditions as respective enzyme solutions except absence of the enzyme.
DNAse and RNAse obtained from the Worthington Biochemical Laboratory, Freehold, N.J.; pepsin obtained from the Nutritional Biochemical Corp., Cleveland, Ohio.

oxyribonuclease (DNAse) (*12*). A concentration of 0.1 mg/ml of DNAse in phosphate buffer completely removed all Feulgen-positive material from the nuclei of the follicle cells and other cockroach tissues within 30 min, but the symbiotes remained Feulgen-positive even after 4 hr exposure to the enzyme. To eliminate the possibility of interfering substances staining with Feulgen, free aldehydes and acetals were blocked by hydroxylamine (*13*). The results indicate that the staining of the microorganisms is not due to either of these groups.

Perhaps the failure of DNAse to remove the Feulgen-positive material is a result of the inaccessibility of the DNA which is protected or masked in some way by other cellular constituents. This possibility has been explored by pretreatment of sections with ribonuclease (RNAse) (*14*), acid hydrolysis, saliva and pepsin followed by DNAse. These experiments and the results are summarized in Table 1. The resistance of the DNA of the microorganisms to DNAse was unaffected by predigestion with RNAse, saliva, and acid hydrolysis. However, pretreatment of sections with 2.0 mg/ml of pepsin for 45 min at 37°C was sufficient to allow removal of the Feulgen-positive material from the microorganisms by DNAse. Sections in the DNAse control solution (that is, the same solution and conditions minus the enzyme) and sections in the pepsin control were hydrolyzed and stained simultaneously with the DNAse-treated slides. The nuclei of the follicle cells were considered the control for the activity of DNAse and the other enzymes and reagents.

In conclusion, the symbiotic microorganisms of the cockroach contain DNA as revealed by the Feulgen reaction. Unlike the nuclei of the cockroach tissues the DNA of the symbiotes in the oocytes and mycetocytes is resistant to digestion by DNAse without previous exposure to the proteolytic enzyme pepsin.

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A Fungus Disease in Clam and Oyster Larvae

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The improved method for growing lamellibranch larvae developed at the U.S. Fish and Wildlife Service laboratory, Milford, Conn., has offered many new possibilities and approaches for studying the behavior and physiological and ecological requirements of these organisms (*1, 2*). Using this method, the larvae of more than 15 species have been successfully cultured through metamorphosis at our laboratory. Of these, however, the larvae of the common clam, *Venus mercenaria,* and of the American oyster, *Crassostrea virginica,* have been most widely studied (*3, 4*).

Clam larvae are especially suitable for experimental work, and usually their cultures are carried through metamorphosis without experiencing undue difficulties. On several occasions, however, some cultures of an experimental series would show a heavy mortality unrelated to the experimental treatment. Recently, while examining such a culture of clam larvae, Davis noticed an organism, which was tentatively identified as a fungus, in many of the dying and dead larvae. Loosanoff and Davis later observed the same or a related fungus in larvae of *C. virginica, Venus mortoni,* and the hybrids of *V. mortoni* ♀ crossed with *V. mercenaria* ♂ and *V. mercenaria* ♀ *crossed* with *V. mortoni* ♂.

The presence of fungus in our larval cultures appears to be of endemic nature. Usually there are only

a few infected larvae in many of the cultures, yet only in rare instances does the disease acquire epidemic proportions, involving the majority of the larvae, and within 2 to 4 days killing almost the entire population.

During the outbreak of the epidemic, many larvae may be observed in various stages of disintegration, with the fungus quite apparent in their interior. Usually several species of flagellates and ciliates invade the dead or dying larvae to feed upon their tissues, leaving, within a short time, empty shells with practically no traces of the fungus. Even in the cultures in which mortality is especially severe, there usually are some larvae that live through the epidemic without contracting the disease, which suggests the possibility that they may be immune to the fungus.

Larvae of all ages, from the very early free-swimming stage to those ready to metamorphose, have been seen parasitized by the fungus. Individuals that had already metamorphosed into juvenile clams have also been found heavily infested on several occasions, and release of zoospores from the fungus in such animals was observed.

At present we do not know the optimum temperature or salinity for the growth of the fungus or the limits it can withstand. Thus far, the larval cultures in which the fungus infection was noticed have been kept within a temperature range of 19.0 to 27.0°C and in a salinity of about 27.0 parts per thousand. Nor do we know whether some infected larvae may survive the infection, recover from it, and later proceed with normal development. It seems, however, that the majority of the larvae stop growing and die soon after they become infected.

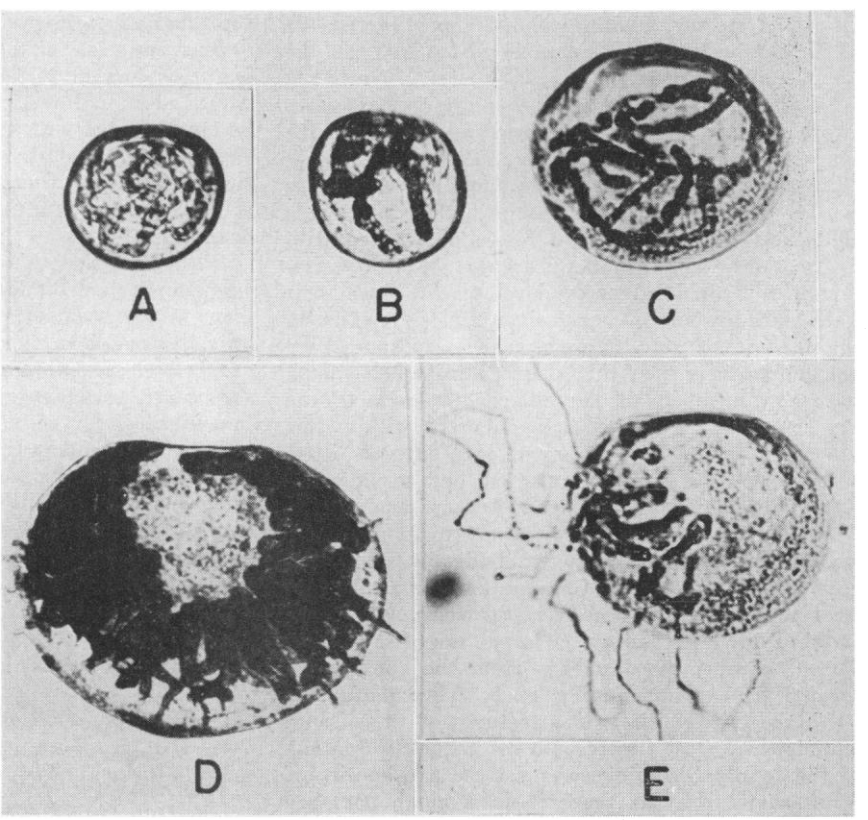

Fig. 1. Larvae infested with fungus. *A* and *B*, young oyster larvae; *C*, young clam larva; *D*, old clam larva; *E*, young clam larva. Larvae *A*, *B*, *C*, and *E* are stained with neutral red, larva *D*, with cotton blue (×224).

The fungus is transmitted by zoospores which emerge through the tip of the exit tube that the sporangium puts forth to the exterior of the infected larva. The zoospores are released in an intermittent stream from these openings, and for a short time they are seen swarming near the edges of the shells of the larvae from which they were released but soon disperse through the water. Theoretically, densely crowded larval cultures should present a better opportunity for development of fungus epidemics than field conditions, because of the proximity of the larvae to one another and, hence, the difficulty of their escaping infection by the large numbers of zoospores swarming about in the water.

Even in this preliminary phase of our investigations, we find that the nature and development of the fungi responsible for this destructive disease show certain distinctive and significant features. Within the larvae, the fungus develops as a contorted, looped, and sparsely branched thallus of torulose character with constrictions at intervals between the swollen and often lobed segments. In both oyster and clam larvae, the thallus may be limited in extent, occupying a relatively small part of the shell cavity, so that its form and structure can be readily seen (Fig. 1*A*, *B*, *C*).

In contrast, in many of the specimens of invaded clam larvae examined, there is much more extensive growth, the fungus occupying a large proportion of the shell cavity with its lobes or segments so densely crowded together in a radiate arrangement that the details of thallus organization cannot be discerned with certainty (Fig. 1*D*). It has not yet been determined whether this difference in thallus character indicates that the simpler, less extensive growth represents a younger phase which will later develop into the more elaborate one or signifies that two different species of fungi are concerned.

As the fungus rapidly develops, the segments mature into sporangia, each with an exit tube extending to the exterior, as can be seen in Fig. 1*D*. The zoospores, already fully developed, emerge through these tubes into the surrounding water. In some larvae, the tubes may protrude considerable distances from the larval shells (Fig. 1*E*).

No sexual stage of the fungus or any resting spores or other resistant entities have been seen in any of the many specimens examined.

Although the identity of the fungi concerned has not yet been specifically determined, their taxonomic affiliations are sufficiently clear to permit tentative designation. Since their thalli are simple in organization with limited extent of development, since their walls show a weak but conclusive cellulose reaction to chloroiodide of zinc, and since their zoospores are biflagellate, it is clear that these fungi belong among the lower members of the biflagellate series of the Phycomycetes. For generic affiliation within this series, the genus *Lagenidium* at once suggests itself both because the looped, contorted, and torulose nature of the thallus is similar and because one species, *Lagenidium calinectes,* is known to occur in salt water and to cause similar destructive epidemics among the larvae of the blue crab in Chesapeake Bay (*5*). In contrast to *Lagenidium,* however, the zoospores in the fungi of our clam and oyster larvae are already fully developed when they emerge from the sporangium through the exit tube and are ready to swim away.

All the critical distinctive features of these fungi affiliate them most closely with the genus *Sirolpidium* of the family Sirolpidiaceae. Yet the two species of *Sirolpidium* thus far recognized, although both are marine and parasitic, have been reported only on such algae as Bryopsis and Ceramium and have never been found on animal hosts. Therefore, the indications are that the fungi attacking the larvae of clams and oysters probably represent one or perhaps even two new species.

It was shown by Loosanoff that the fungus acquires a deeper stain than larval tissues if living larvae are kept in a solution of neutral red in sea water. Thus, this stain can aid in detecting the first appearance of the fungus, and also, by "tagging" the infected larvae, we can study the progress of the epidemics, the percentage of survival of fungus-affected individuals, and other pertinent features. Neutral red may also prove valuable in staining mass samples of natural plankton for easier examination and for determining whether some of the organisms constituting it are infested with the fungus. The usual cotton blue lactophenol technique can also be used to demonstrate presence of the fungus in preserved samples.

In experiments at the U.S. Fish and Wildlife Service laboratory at Milford, we are attempting to find which additional species of lamellibranch larvae may become infected with the fungus disease. At the same time, we are carefully examining the internal condition of the larvae found in our field plankton samples to ascertain whether the fungus occurs under natural conditions.

We believe that this is the first report of a fungus parasite in lamellibranch larvae. Since only a few cases of the occurrence of parasitic fungi in marine animals are known, this discovery should be of considerable biological interest. The possibility exists that the occurrence of an epidemic of this fungus among lamellibranch larvae in nature may seriously affect the sequence of events in the food chain. It is also possible that if we develop a method for control of the fungus under hatchery conditions, a problem on which we are working at present, the artificial cultivation of clams, *Venus mercenaria,* and other mollusks may be rendered commercially feasible.

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