SAPROLEGNIA DICLINA HUMPHREY

AS A PARASITE OF THE SALMONID,

ONCORHYNCHUS KISUTCH


by


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## ABSTRACT

Studies of Saprolegnia infections of fish in British Columbia were
made to determine disease causing agents and infection conditions.

Saprolegnia diclina Humphrey was the most frequently observed para-
site. This fungus reproduced sexually both on fish tissue and hemp seed
cultures. No definite isolations of S. parasitica Coker were made although
some non-sexually reproducing isolates of a Saprolegnia sp. were found.
The validity of the species, S. parasitica, has been examined and questioned
on the basis of present identification characteristics.

Infection studies using S. diclina as the parasite and fingerling
coho (Oncorhynchus kisutch) as the host indicated a distinct correlation
between temperature and infection. At normal cool temperatures, e.g.,
$8^{\circ}$C, no infection occurred; at $9^{\circ}$C or above, some infection resulted.
Above $9^{\circ}$C, the rate of infection increased as temperature increased.
Temperature was also associated with the time at which infection occurred
after inoculation. At $18^{\circ}$C, infection began earlier than at $13^{\circ}$C. Heat-
shock treatment tended to reduce the temperature-time effect causing
initial infection at $13^{\circ}$ and at $18^{\circ}$C to occur almost simultaneously.
Cold-shock treatment resulted in some infection. Such treatment, however,
did not produce the same immediate infection as heat-shock.

Histological studies demonstrated the infection to be concentrated
in the host epidermis with fungal hyphae at sites of heaviest infection
extending through the dermis and into underlying muscle tissues.

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## ACKNOWLEDGEMENT

INTRODUCTION AND HISTORICAL RÉSUMÉ

Saprolegniaceous infections of fish and other aquatic animals have attracted the interest of biologists since Spallanzani's first report in 1777 of "molds" growing on minnows and leeches (cf. Ramsbottom, 1916). Most investigations have, however, aimed at fungus control with little attempt to identify the fungi involved or to clarify the conditions under-lying infection. Fisheries biologists, on the whole, have shown little concern for fungus disease as most consider it a secondary infection attacking only diseased or dying fish. Furthermore, Saprolegnia and other Phycomycetous parasites of fish and fish eggs are relatively easily controlled by removal of infected organisms or by standard chemical treat-ments (Hoffman, 1963). These fungus infections, however, have at times aroused serious concern. High mortality rates among fish, especially Salmonids, suffering from fungus infection were early reported in England and Scotland (Huxley, 1882 a, b, c; Murrary, 1885; Clinton, 1893; Patterson, 1903) and in the United States (St. George and Barron de la Valette, 1884; Koltz, 1883; Valery-Mayet, 1885; Rutter, 1904; Rosenberg, 1908). Whereas such early reports were mainly observational, some of the more recent studies have aimed at identification of fungi and hosts with attempts to clarify the host-parasite relationship (Kanouse, 1932; Tiffney, 1937, 1939 a, b; Shanor and Saslow, 1944; Vishniac and Nigrelli, 1957; Wolf, 1958; Scott and O'Bier, 1962; Egusa, 1963, 1965, a, b).

One of the most complete historical résumés of "fish mold" (Rucker, 1944) indicates that, although the literature on such fungus diseases is voluminous, our understanding of the problem is limited. This paradox is partially explained by the fact that so many different

groups of people have studied these diseases. Botanists, zoologists, fisheries biologists, pathologists, and bacteriologists have all examined the problem from their own various points of view. The result has been many contradictory reports and many incomplete or invalid descriptions.

With the exception of the many papers on control measures, the literature covers three general topics: hosts attacked, identification of the fungi involved, and etiology of the diseases themselves. Reports of fungi on almost all groups of Teleostei, as well as several members of the Amphibia, indicate an extremely wide host range (Tiffney, 1939 a). It has also been shown, however, that certain species are more susceptible to fungus attack than others (Tiffney, 1939 a). During Tiffney's experiments, attempts were made to infect several species of 10 different families of fish with the fungus, Saprolegnia parasitica Coker. Members of the Siluridae, Catostomidae, and Salmonidae were among the most susceptible to fungus attack. Only Aguilla chrysypa of the Anguillidae resisted infection.

Identification of "fish molds" has been complicated by several factors - failure of certain isolates to reproduce sexually (a primary requirement for valid identification); incomplete descriptions; and inadequate knowledge of potential species variability of the involved fungi. Probably the greatest confusion arises from the fact that the fungus most frequently observed as a fish parasite, S. parasitica, seldom produces the sexual structures necessary for positive identification.

As is shown in Table 1, a number of species from several genera of aquatic Phycomycetes have been reported as parasites of fish and other

## TABLE 1

### SAPROLEGNIACEAE REPORTED AS PARASITES OF AQUATIC ANIMALS

| Fungus | Reference |
|---|---|
| Achlya ambisexualis J.R. Raper, ♂ and ♀ | Vishniac & Nigrelli, 1957 |
| A. bisexualis Coker & A. Couch, ♂ and ♀ | Vishniac & Nigrelli, 1957 |
| A. colorata Pringsheim (as A. racemosa var stelligera cornu) | Humphrey, 1893 |
| A. flagellata Coker | Tiffney & Wolf, 1937 |
| 1 A. nowickii Raciborski | Walentowicz, 1885 (see Tiffney, 1939 a) |
| A. polyandra Hildebrand | Hine, 1878 (see Tiffney, 1939 b) |
| A. prolifera C.G. Nees | Schnetzler, 1887 (see Tiffney, 1939 b) |
| A. klebsiana Pieters | Vishniac & Nigrelli, 1957 |
| 2 A. sparrowii Reischer | Vishniac & Nigrelli, 1957 |
| Achlya sp. | Vishniac & Nigrelli, 1957 |
| Aphanomyces laevis de Bary | Shanor & Saslow, 1944 |
| Ap. astaci Schikora | Prowse, 1954 |
| Ap. daphniae Prowse | Prowse, 1954 |
| Ap. hydatinae Valkanov | Valkanov, 1931 |
| Ap. ovide struens Gicklhorn | Prowse, 1954 |
| Calyptralegnia achlyoides (Coker & Couch) Coker | Vishniac & Nigrelli, 1957 |
| Dictyuchus monosporus Leitgeb | Tiffney, 1939 b |
| Leptolegnia baltica Hohnk & Vallin | Hohnk & Vallin, 1953 |
| L. caudata de Bary | Coker, 1923 |
| L. marina Atkins | Atkins, 1954 |
| Protoachlya paradoxa Coker | Vishniac & Nigrelli, 1957 |
| 3 Saprolegnia delica Coker | Vishniac & Nigrelli, 1957 |
| S. diclina Humphrey | Rioux & Achard, 1956 |
| S. ferax (Gruith) Thuret | Smith, 1878 (see Tiffney, 1939 b) |
| 4 S. invaderis Davis & Lazar | Davis & Lazar, 1940 |
| S. megasperma Coker | Vishniac & Nigrelli, 1957 |
| 4 S. monoica Pringsheim | Walentowicz, 1885 (see Tiffney, 1939 a, b) |
| 4 S. mixta de Bary | Clinton, 1894 (see Tiffney, 1939 b) |
| S. parasitica Coker | Coker, 1923 |
| 5 S. torulosa de Bary | Huxley, 1882 a |
| Thraustotheca clavata (de Bary) Humphrey | Vishniac & Nigrelli, 1957 |
| T. primoachlya Coker & Couch | Vishniac & Nigrelli, 1957 |

1 Excluded taxon (Johnson, 1956).
2 Reduced to synonymy with A. racemosa (Johnson, 1956).
3 Reduced to synonymy with S. diclina (Seymour, 1966).
4 Reduced to synonymy with S. ferax (Seymour, 1966).
5 Taxon of doubtful affinities (Seymour, 1966).

aquatic animals. In fact, Vishniac and Nigrelli (1957), using the Mexican platyfish, Xiphophorus maculatus, as their host, found that under various experimental conditons, 15 of the 17 species of Saprolegniaceae which they used in their study were potential parasites.

Descriptions of these fungus diseases and their effects on fish are extremely inconsistent. Fungi have been reported to develop only on previously injured, lesioned, or otherwise damaged tissues (Plehn, 1924); at other times, they have been reported on apparently healthy tissues (Huxley, 1882 a). Some fish reacted by vigorously rubbing infected areas (Huxley, 1882 a; Stirling, 1880; Tiffney, 1939 a); others showed no direct response to fungal growth and appeared unaware of its development.

Contradictions, however, are most striking in discussions of the conditions leading to infection of fish with fungus. Such fungus diseases have been attributed to overcrowding, pollution, and reduction of water supply (Brook, 1879); to a rise in temperature (Cummins, 1954; Scott and O'Bier, 1962); to a nutritional deficiency (Jewell, Schneberger and Ross, 1933); to mechanical injury (O'Donnell, 1941; Hoffman, 1949); and to predisposing bacterial infections (Hardy, 1911; Davis, 1923; Rucker, 1944; Egusa, 1965 a, b). For almost every suggested condition promoting infection, a contradictory argument may be found. In contrast to Cummins' and Scott's views supporting a rise in temperature as the necessary condition, Davis (1923) felt low temperatures were necessary for infection. Murray (1885) felt temperature was of little importance except in extremes. Rucker (1944), opposing the theory of mechanical injury, cited several instances (Edington, 1889; Heacox, 1941) when deliberate mechanical injury failed to result in fungus attack. His theory of a primary bacterial

infection, however, finds a contradiction in the studies of Vishniac and
Nigrelli (1957). These investigators found no evidence of bacterial in-
fection in thin sections of infected tissues.

In all the confusing array of arguments, the only consistency seems
to be the general feeling that Saprolegniaceous fungi will not attack
healthy fish living under normal conditions. Most reports suggest the
fungus is a secondary consequence of some other primary, pathogenic con-
dition or agent.

The cause of death once fungus infection is established has also
been a debatable point. Tiffney (1939 a) suggested three possible causes:
mechanical destruction of tissues, production of toxins, and ionic upset
due to surface tissue destruction. His earlier work (1936) indicated the
presence of heat stable exotoxins and endotoxins. Rucker (1944), on the
other hand, was unable to find positive evidence of any toxic material
produced by the fungus. Occasionally, reports of death from suffocation
caused by fungus on gill tissues appear (Brook, 1879). These, however,
are rare and most investigators, e.g., Rucker (1944) and Tiffney (1936),
indicate that soffocation had no part in the cause of death of the fish
examined.

From the above discussion, it is evident that the problem of Sapro-
legniaceous fungus infections of fish demands much further experimental
study. The problem becomes even more acute when we consider some of the
economic factors involved. In Japan, serious outbreaks of such a fungus
disease have threatened the eel-farming industry for the past 10 years.
The disease, most serious in April and May, has caused severe losses in

pond-reared eel populations (Egusa, 1963, 1965 a, b). Egusa's experimental studies on infected eels suggest a primary bacterial infection. Many aspects of the disease, however, are still poorly understood and further studies are presently being carried out. In the western United States, Rucker (1944) carried out an extensive study in the Columbia River system of chinook (O. tshawytscha), sockeye (O. nerka), and steelhead (Salmo gairdnerii gairdnerii). Serious fungus outbreaks had been encountered among these fish during transplanting operations prior to construction of the Grand Coulee Dam.

Similar problems have recently prompted some concern in Western Canada. With the development of several large scale, artificial spawning channels (MacKinnon, 1961), fungus infections have become increasingly troublesome. An example of such an area is the Robertson Creek Spawning Channel near Port Alberni, British Columbia. Here fish are held in downstream spawning channels constructed to provide the optimum conditions for spawning Pacific salmon -- mainly coho, pink (O. gorbuscha), and sockeye -- and for rearing their young (Lucas, 1960). Fungus infections among many of the fish are often rampant with high concurrent prespawning mortality rates. In 1965, for instance, losses of both coho and sockeye prespawners exceeded 40% (Kearns, 1965).

Such mortalities are not uncommon among prespawning salmon. Royal and Seymour (1940) noted a 57% mortality among planted sockeye salmon of the Puget Sound area which they attributed to "...fungus growth on the head and especially on the snout and eyes."

At Robertson Creek, the fish are often badly bruised from continual

attempts to move past gates to natural upstream spawning beds. Many fish are heavily infected with fungus. The white mycelial mats of fungus often extend over 50% of the body surface. In some years, however, mechanical injury has not appeared serious enough to account for prespawning mortalities approaching 50% and a bacterial infection was suspected. Although some fish had lesions characteristic of Chondrococcus columnaris infections, no serious build up of the disease could be established from later bacteriological studies.

As mortality rates were often higher at temperatures around or above 13°C, it was felt they may have been temperature associated (Kearns, 1965).

In the following study, an attempt has been made to test the hypothesis that temperature may be a critical factor in predisposing fish, directly or indirectly, to infection by Saprolegniaceous fungi. The fungus infecting fish at Robertson Creek was isolated, identified as S. diclina Humphrey, and used in experimental infection studies on fingerling coho. Considerable time was spent in developing techniques for obtaining single-spore, bacteria-free cultures prior to identification and experimental studies. Fungi from fish and fish eggs collected at several other localities in British Columbia were also isolated and identified.

## MATERIALS AND METHODS

### Collection and Culture

Fungi were collected in the field from living and dead fish and from fish eggs by transferring hyphae directly to Petri dishes of potato-dextrose agar (Difco). On several occasions, fungi were removed directly from fish tissues for immediate microscopic examination.

Repeated transfers using several techniques were carried out to obtain bacteria-free cultures. The following techniques were used with varying degrees of success:

1. Raper's glass ring method (Raper, 1937).

2. Agar wells:

   Small cubes of agar were removed from the centre of medium in Petri dishes and placed on the agar surface adjacent to the well. Fungus was inoculated at the bottom of the well and hyphal tips subsequently transferred from the surface of the agar cube.

3. Rose bengal:

   35 mg/l and 17 mg/l were added to plates of various types of agar media.

4. Potassium tellurite (Willoughby, 1962):

   Fungi were grown on potato-dextrose agar containing 100 mg/l potassium tellurite. A second treatment was used in which fungi were grown in 125 ml. Erhlenmeyer flasks containing

liquid medium - .025% maltose and .025% peptone in distilled, carbon-filtered water (Kanouse, 1932). To prepare flasks, 150 mg maltose and 150 mg peptone were autoclaved in 49 ml. water. 1 ml. potassium tellurite solutions were later added to each flask of cooled medium bringing total medium per flask to 50 ml. A replicated series of flasks was prepared containing concentrations of potassium tellurite ranging at small intervals from 1.0% to 0.002%. Two discs of fungus inoculum were placed in each flask. Inoculum discs were cut with a #3 cork borer from bacteria contaminated cultures growing on potato-dextrose agar. Flasks were examined over a five day period for growth of fungi and bacteria. Bacteria-free cultures remained clear; contaminated ones turned murky.

5. Actidione:

500 mg/l was added to potato-dextrose or to corn meal agar (B-B-L), autoclaved, and poured into Petri dishes.

6. Chloramphenicol:

50 mg/l was added to the medium. Medium was prepared as in treatment 5.

7. Penicillin-G:

Liquid antibiotic was added in one of two concentrations (190 mg/l and 125 mg/l) to autoclaved potato-dextrose agar. The medium was poured into Petri dishes.

8. Penicillin-G and streptomycin-sulfate:

Antibiotics (190 mg/l penicillin-G and 100 mg/l strepto-mycinsulfate) were added in the dry form to autoclaved potato-dextrose agar. The medium was poured into Petri dishes.

9. Silver-ring method (Powell & Tenny, 1964):

To test the oligodynamic effect of silver ions on con-taminated cultures, a silver ring (3.5 mm in diameter and 1.5 mm in height) was placed over the depression of a Shoemaker culture slide (Clay-Adams). The slide and ring were placed in a Petri dish and autoclaved. Sub-sequent treatment followed that for Raper's glass-ring method.

10. Ultra-violet light (Blank & Tiffney, 1936):

Plates of levulose-peptone agar medium were prepared and irradiated as outlined by Blank and Tiffney. The lamp used was a General Electric Germicidal, 30 watt. Irradi-ated plates were inoculated with fungal discs removed from cultures grown on potato-dextrose agar. Cultures were examined and compared with non-irradiated control plates for growth rate of fungi and presence of bacteria.

11. Low temperature:

Potato-dextrose and corn meal agar cultures were incu-bated at $5^{o}C$ for a two week period. Control cultures were incubated at $13^{o}C$.

For all of the above techniques, cultures were tested for presence of bacteria by subculturing in liquid maltose-peptone medium. Most techniques were tried individually, in various combinations with each other, and in series. Numbers one and two were combined with three, four, and seven respectively, and number ten with three, four, six, and seven.

Single spore cultures were obtained by growing fungi on hemp or sesame seeds in distilled carbon-filtered water. Following production and release of motile spores by the fungus, some of the water containing the spores was sprayed onto plates of potato-dextrose agar. A fine spray was obtained by using a fine glass atomizer connected to a tank of compressed air. Hyphae from individual germinating spores were subcultured for identification.

Stock cultures were maintained in tubes of oatmeal or corn meal agar incubated at $13^{o}$C. Cultures were transferred every six to eight weeks.

### Identification

Identification of fungi was based on measurements made on sexually mature, hemp seed cultures. A culture of S. parasitica, C.B.S. Meurs, was obtained from the Centraal bureau voor Schimmelcultures, Baarn, Netherland, as a comparative check during identification. Average measurements were made on the basis of 25 observations. References and keys included Humphrey (1893), Coker (1923), Coker and Matthews (1937), Johnson (1956), and Seymour (1966).

Several techniques were tried to induce sexual reproduction in those cultures which failed to produce oogonia and antheridia on hemp seed

cultures. The C.B.S. culture of S. parasitica, a local non-sexually reproducing culture, and a culture of S. diclina (isolate D38) were grown in flasks of dilute peptone-leucine or dilute peptone-maltose liquid medium using a technique introduced by B. Kanouse (1932).

Cultures of S. parasitica and S. diclina (D38) were treated with two steroids and with three raw oils, substances found to stimulate sexual reproduction in certain Phythium spp. (Haskins, et al., 1964). Fungi were grown initially on plates containing 25 ml solid MSM medium (personal communication, R.J. Bandoni, 1967). The ingredients and method of preparation for MSM medium are given in the Appendix. Discs of inoculum were subsequently transferred to 125 ml Erhlenmeyer flasks containing 50 ml liquid MSM medium. Following 24 hours growth in these flasks, fungal discs were transferred to flasks containing a modified MSM medium prepared without the addition of dextrose and casein. Individual steroids or oils were placed in this second set of flasks. The steroids, cholesterol and sitosterol, were dissolved in 95% ethyl alcohol. Stock steroid solutions were prepared such that 1 ml of stock solution added to 50 ml of medium resulted in steroid concentrations of 1, 5 or 10 ppm. The raw oils -- linseed, olive, and corn oil -- were added directly to flasks with a 2 ml pipette. One, two, or three drops were added to each flask. Control flasks contained 1 ml 95% ethyl alcohol. All cultures were examined regularly over a three week period for the presence of oogonia and antheridia.

## Infection Studies

### Experiments demonstrating the effect of temperature increase:

Experimental fish tanks were set up as is shown in Plate 1. Three glass aquaria ($9" \times 6" \times 7"$) were suspended in each of the three larger wooden tanks. Water temperature in each wooden tank was maintained by a thermostatically controlled heater working against a continuous supply of cold water. Tanks I, II, and III were maintained at 8, 13 and $18^{o}$C respectively in experiments I, II, and III. During experiments IV, V and VI, the temperature in tank I ranged between 9 and $10^{o}$C. A constant 18 hour light-day was regulated by 60 watt bulbs set in wooden hoods over each tank. Tin foil, covering half the surface of each aquarium, allowed fish to move freely between light and shade. Four liters of autoclaved, dechlorinated water were placed in each aquarium. Aeration was supplied by individual air-stones.

Experimental fish, fingerling coho, seined in Little Campbell River, British Columbia, were all of the same size range (averaging 3.5 cm from snout to end of caudal peduncle) and of the same age group. Freshly captured fish were held in large stock tanks where they were given at least a seven day period to adjust to the laboratory conditions. Water temperature in stock tanks was $8^{o}$C. Prior to introduction to experimental aquaria, fish were removed from stock tanks and held in a separate container at room temperature to allow a gradual increase in water temperature to that of the experimental aquaria. Four fish were introduced into each of the nine aquaria.

Fungus inoculum was prepared from cultures of $\underline{S}$. $\underline{diclina}$, isolate D38. Single agar discs cut from cultures grown in Petri dishes containing

PLATE I

Apparatus for fish infection studies

Figure:

    (1)  Experimental tanks.

          O: outlet for continuous water supply to main tank;

          H: hood with built-in light fixture.

    (2)  Aquaria set in tanks during infection study.

          T: tank;

          A: glass aquarium containing four fingerling coho;

          TH: thermostat for heating unit;

          AH: air hose;

          F: foil cover;

          I: water inlet.

PLATE 1
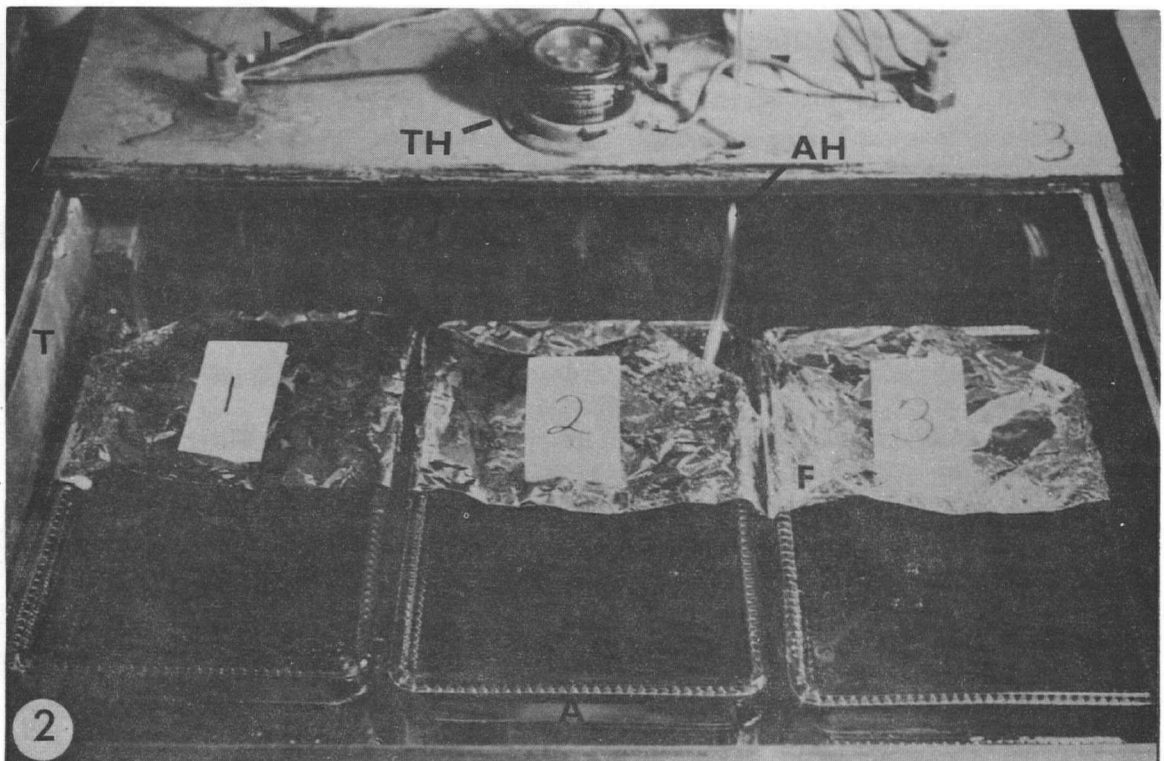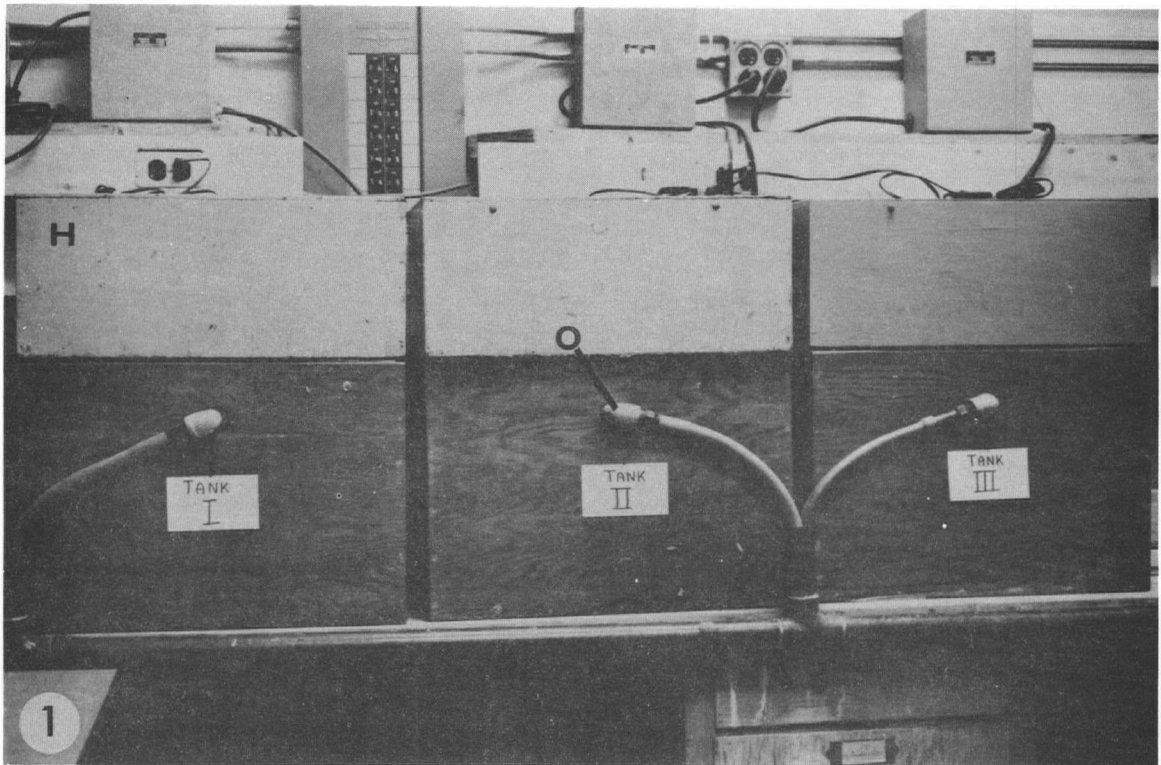
25 ml solid MSM Medium were transferred to 125 ml Erhlenmeyer flasks containing 25 ml liquid MSM medium. After 24 hours, fungal discs were transferred to small Petri dishes containing 15 ml sterile, distilled carbon-filtered water. These cultures, following 12 hours incubation, were inoculated directly into 2 of the 3 aquaria in each tank. The third and uninoculated aquarium acted as a control. Fish introduced on day 1 were exposed to fungus on day 3 and on every third day thereafter until day 18 when the experiment terminated. During the 18 day period, fish were fed daily with frozen brine shrimp and observed closely at approximate 12 hour intervals. At each observation, water temperature, general condition and behaviour of fish, and development of fungus or other infections, were recorded. All dead fish were removed and preserved in 10% formalin or in Bouin's solution. A positive infection was recorded only if fungus was observed growing on the tissues of a living fish. For each experiment, fungus on a few fish was re-isolated and identified. All remaining fish at the close of an experiment were preserved in formalin and examined microscopically both externally and internally for fungus or other infections. The experiment was run in triplicate.

Experiments demonstrating the effect of temperature shock:

The procedure followed that for the previous temperature experiments with the following changes. Four tanks each containing three aquaria were maintained at 3.5-4.5$^{\circ}$, 9-10$^{\circ}$, 13$^{\circ}$, and 18$^{\circ}$C, respectively. Water temperature in the 3.5-4.5$^{\circ}$ tank was controlled by a thermostatically controlled copper, cooling coil. Fish were transferred immediately from stock tanks to experimental aquaria thus subjecting them to a sudden rather than gradual temperature change. The initial inoculum was added to aquaria on

day one rather than on day three.

## Histological Techniques

Several fish, immediately upon death from fungus infection, were fixed in Bouin's solution. Paraffin sections were prepared and stained by the periodic acid-Schiff technique combined with a light green counter-stain (Emmons, et al., 1963). Other techniques used were a variant of Mallory's using haematoxylineosin with aniline crystal violet (Lillie, 1954) and a cresyl fast violet stain (Puchtler and Sweat, 1964).

## RESULTS

### Collection and Identification

Table 2 summarizes collection sites, hosts and fungi isolated during this investigation. Sexual reproduction was observed on all cultures except D1. The most common parasitic species was S. diclina which occurred at all collection sites except Adams River. The Adams River fungus plus two Genesse Creek isolates, D26 and D28-G4b, keyed out as S. diclina. However, oogonia and antheridia were much slower in development and much fewer in number than those of other cultures of S. diclina. The fine often wavy hyphae closely resembled that of the C.B.S. culture of S. parasitica. Further study is necessary for positive identification of these three isolates.

Examination of S. diclina removed directly from fish tissue revealed several interesting morphological features. Whereas in hemp seed culture, the fungus produced typical Saprolegnioid sporangia with free swimming primary zoospores, on fish tissue, it produced masses of cylindrical, aplanoid sporangia (Plate 2, figure 4). Many such sporangia were observed with the long tubes of germinating spores projecting from all sides. At no time was the release of primary, free-swimming zoospores observed. On one occasion, S. diclina was found producing oogonia and antheridia on fish tissue. Plate 2 illustrates the morphology of S. diclina, isolate D40 as it appeared directly after removal from the tissues of a live, adult coho. All isolates of S. diclina produced masses of oogonia, antheridia, and mature oospores in hemp seed culture. Oospores were not typically centric as described by Coker (1923) or

TABLE 2:  Collection data and identification of fungi isolated

| Collection Code No. | Date | Locality | Host | Host Condition | Identification |
|---|---|---|---|---|---|
| D1 | 14/6/66 | Hatchery, U.B.C. | Gasterosteus aculeatus | living | Saprolegnia sp. |
| D3 | 27/6/66 | " " | Salmo salar | dead | Achlya oblongata |
| D4 | 27/6/66 | " " | Oncorhynchus gorbuscha | dead | S. diclina |
| D51 | 15/5/67 | " " | Eggs, G. aculeatus | dead ? | S. diclina |
| D6 | 12/9/66 | C.B.S., Baarn, Holland | -- | -- | S. parasitica |
| D7 | 30/9/66 | Fisheries Research Bd., Nanaimo, B.C. | Eggs, O. nerka | dead ? | S. diclina |
| D41 | 1/11/66 | " | Eggs, O. gorbuscha | dead ? | S. diclina |
| D8, D9 | 6/10/66 | Robertson Creek, B.C. | O. kisutch (prespawner) | living | S. diclina |
| D10 | 6/10/66 | " " | O. gorbuscha (prespawner) | living | S. diclina |
| D34, D35 | 21/10/66 | " " | O. nerka | dead | S. diclina |
| D37, D38, D40 | 21/10/66 | " " | O. kisutch | living | S. diclina |
| D39 | 21/10/66 | " " | O. kisutch | dead | S. diclina |
| D43, D44, D45, D45a | 16/5/67 | " " | water samples | -- | S. diclina |
| D46, D47, D49b | 16/5/67 | " " | water samples | -- | Saprolegnia sp. |
| D23, D24 | 9/10/66 | Adams River, B.C. | O. nerka | dead | Saprolegnia sp. |
| D26, D28-G4b | 29/9/66 | Genesse Creek, B.C. | O. nerka (spawned) | dead | Saprolegnia sp. |
| D28-G3 | 29/9/66 | " " | O. nerka (spawned | dead | S. diclina |

Johnson (1956). The ooplasm, placed slightly to one side, was surrounded by several layers of oil droplets, up to three on one side and five on the other (Plate 3, figure 11). In all other characteristics, the fungus coincided with Coker's description of S. diclina (Coker, 1923). Plate 3 illustrates the morphology of S. diclina, isolate D40, in hemp seed culture.

Attempts to obtain sexual reproduction in the type culture of S. parasitica failed for all but one treatment. A few mature oogonia and antheridia developed in three flasks containing one, two, or three drops of corn oil. In a repetition of the treatment, no sexual organs developed.

## Culture Techniques

Attempts to obtain bacteria-free cultures using any single one of the usual techniques proved unsuccessful. Superficially, cultures appeared uncontaminated; however, subculturing in liquid medium demonstrated the presence of bacteria. Microscopic examination of hyphae submerged in agar revealed a mass of motile bacteria swarming in a narrow, liquid band adjacent to hyphal walls. These bacteria proved to be gram negative rods which showed no sensitivity to penicillin.

Treatment with potassium tellurite indicated the bacterial contiminant was far more tolerant to the treatment than were the fungi tested. Bacterial contaminants tolerated up to 0.08% potassium tellurite. Fungal growth was inhibited at all concentrations above .006%.

Bacteria-free cultures were eventually obtained by repeated transfers using the glass or silver ring technique alternately with growth on irradiated medium. Even this method, however, was not consistently suc-

PLATE 2

<u>Saprolegnia</u> <u>diclina</u> Humphrey - morphology on fish tissue:

Figure:

    (3)   Zoosporangium with encysted primary zoospores.

    (4)   Aplanoid zoosporangium with germinating zoospores.

    (5)   Gemmae.

    (6)   Gemma functioning as zoosporangium.

    (7)   Oogonia and oospores.

# PLATE 2



50 μ
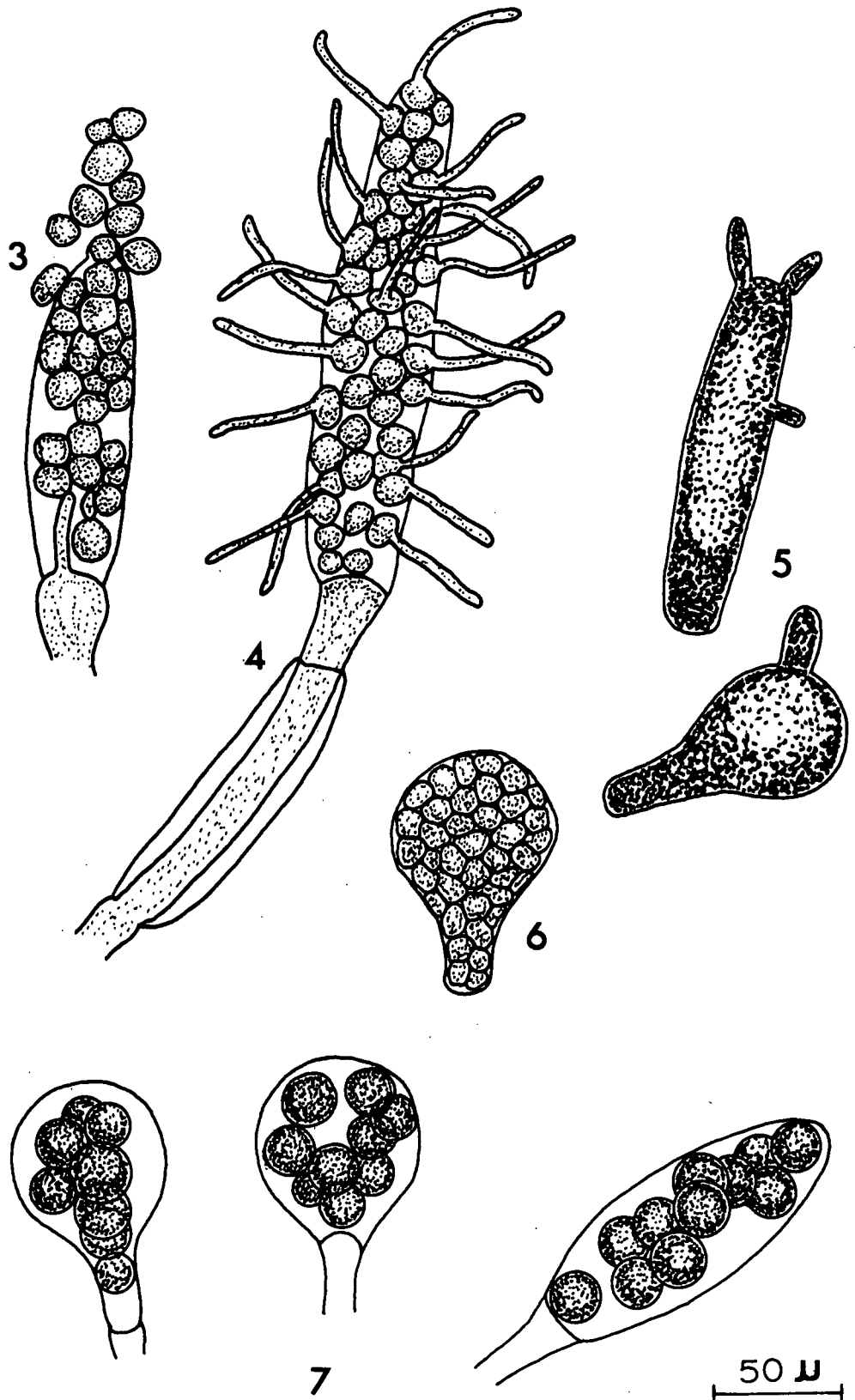
PLATE 3

Saprolegnia diclina Humphrey - morphology on hemp seed culture:

Figure:

(8) Zoosporangium releasing motile, primary zoospores; renewal of zoosporangium by internal proliferation. Scale a

(9) Empty zoosporangium; renewal by side branching. Scale a.

(10) Oogonia, diclinous antheridia, and oospores. Scale a.

(11) Mature oospore showing peripheral rings of oil droplets surrounding ooplasm; position of ooplasm slightly off-center demonstrating variation from recognized centric or subcentric oospore types. Scale b.

# PLATE 3

8

9

10

11

a |—— 50 ᴜ ——|

b |—— 25 ᴜ ——|
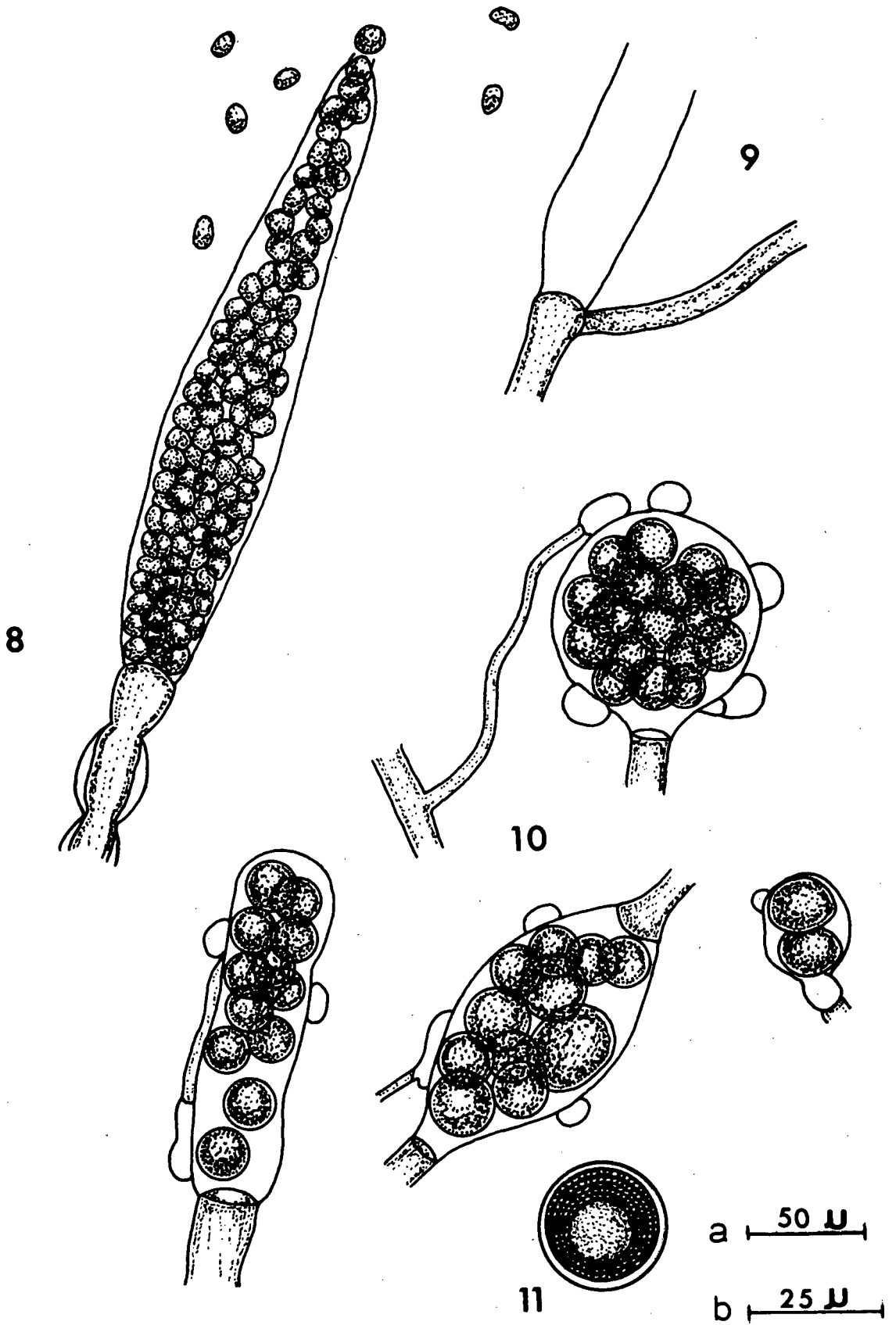
cessful. Many cultures, after long periods of incubation, became contaminated suggesting the presence of extremely resistant bacterial spores.

## Infection Studies

### Effect of temperature increase:

Results from experiments I, II, and III indicated a distinct correlation between temperature and infection of coho with $\underline{S}$. $\underline{diclina}$. The effect of water temperature, summarized for each experiment in Figure 12, was similar in each of the three 18 day periods. Figure 13 gives combined results. Statistical analysis using a Chi-square test demonstrated a significant change in fungus mortality at the three temperature regimes at the 99% confidence level. At temperatures between $8^{o}$ and $9^{o}$C no infection with $\underline{S}$. $\underline{diclina}$ occurred; one control and one experimental fish died of unknown causes. Some fungus infection occurred at both $13^{o}$ and $18^{o}$C. In each experiment, the number of infected fish was greatest at the higher temperature. The difference between infection at $13^{o}$ and $18^{o}$C, however, was not statistically significant. At these temperatures, no fish in the experimental tanks died of causes other than fungus infection. A few control fish died -- some owing to a bacterial infection causing tail rot and some owing to fungus infection. Fungus contamination in these aquaria probably resulted from the transfer of fungus spores from experimental aquaria by splash from air hoses or from jumping fish.

Further analysis indicated a correlation with temperature and the time of infection. Figure 14 illustrates the number of fish infected on each day at $13^{o}$ and at $18^{o}$C. Analysis of this data using the Mann-Whitney u test for non-parametric statistics (Siegel, 1956) indicates a significant

Figure:

    (12)  Effect of temperature increase on infection of coho

        with <u>Saprolegnia</u> <u>diclina</u> during 18 day periods.

FIGURE 12
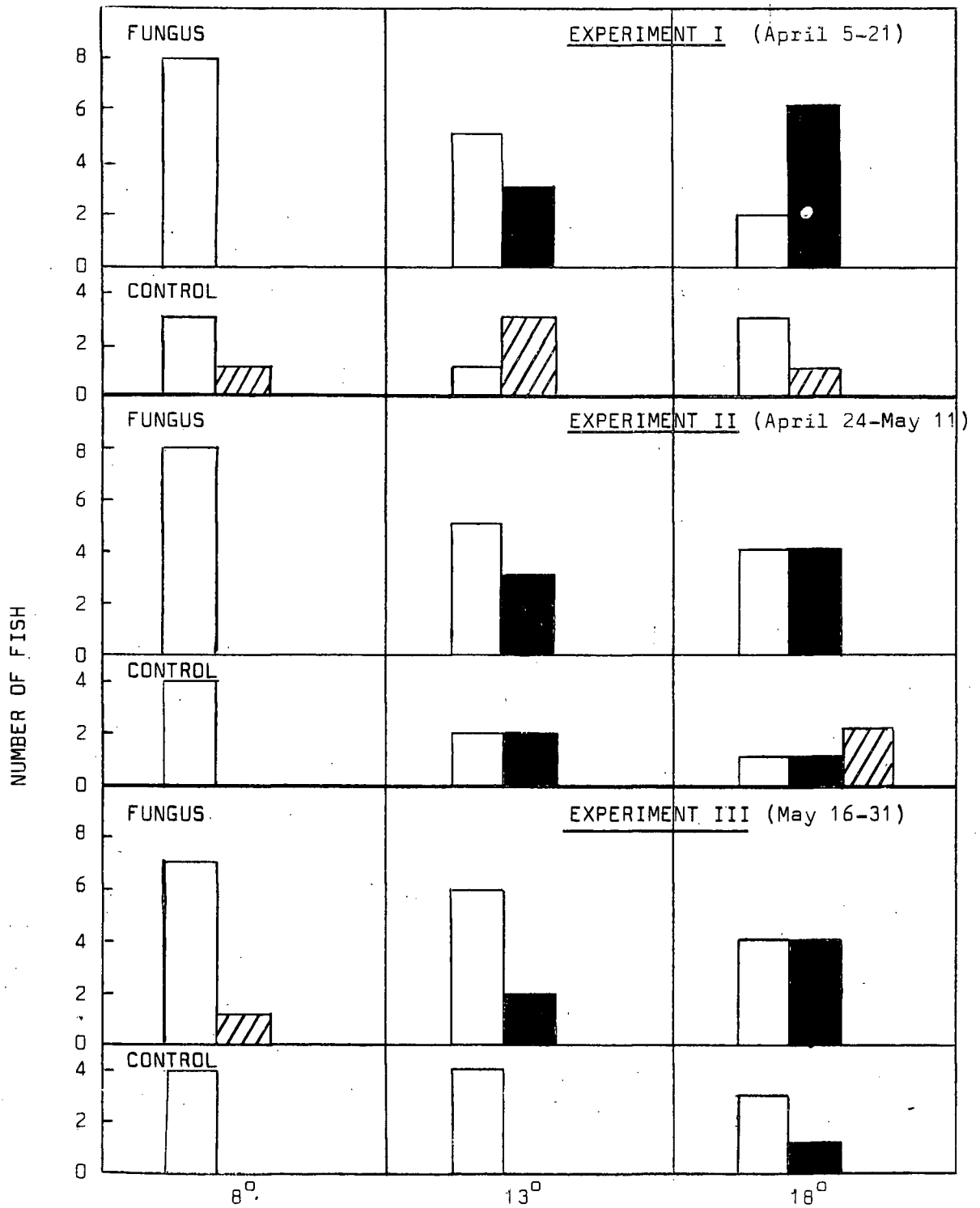
Figure:

    (13)   Effect of temperature increase on infection of coho

             with <u>Saprolegnia diclina</u>. Chart summarizes

             results from experiments I, II, and III.

FIGURE 13



FUNGUS

NUMBER OF FISH

CONTROL

Water temperature (°C)

Fish healthy throughout.

Fish died from fungus infection.

Fish died from causes other than fungus.
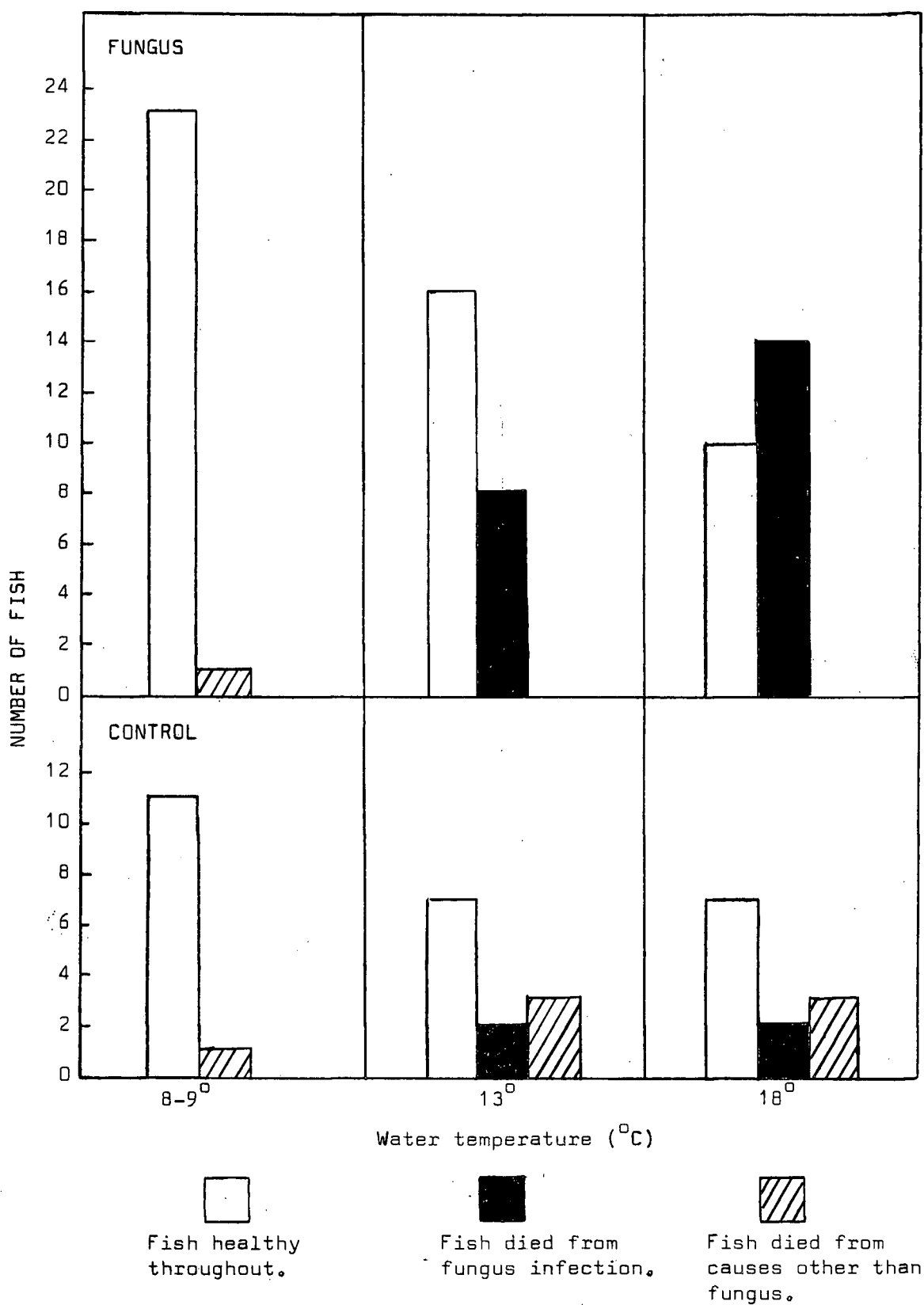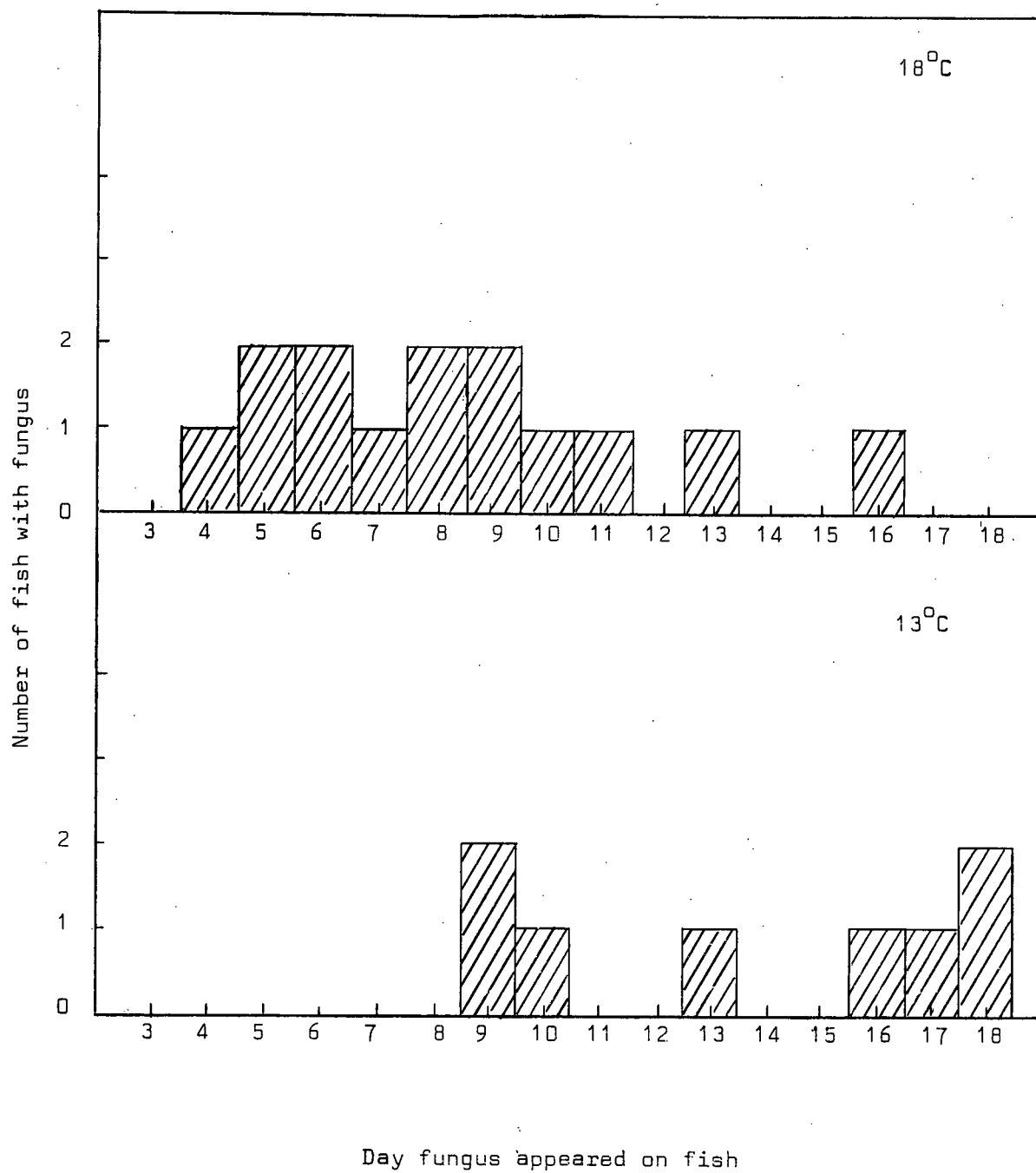
Figure:

(14)  Effect of temperature increase on time of infection

of coho with <u>Saprolegnia</u> <u>diclina</u> during

18 day period.

FIGURE 14



Day fungus appeared on fish

difference at the 99% confidence level between infection times for the two temperatures. At $18^{\circ}$C, the majority of infections occurred within the first seven days while at $13^{\circ}$C infection occurred only during the second week.

### Effect of temperature shock:

Figures 15, 16, 17, 18, and 19 summarize results from experiments IV, V, and VI. In two of the three experiments, fungus infection occurred at all three temperatures. As in experiments I, II, and III, some control fish died of fungus infection and some of unknown causes, possibly bacterial infections. Analysis of data using a Chi-square test revealed no significant difference at the 95% confidence level between the three treatments. The overall effect at $13^{\circ}$ and at $18^{\circ}$C was not significantly different from that of experiments I, II, and III. Some change, however, occurred in the time of infection. Figure 17 demonstrates a leveling out in the times of infection at $13^{\circ}$ and $18^{\circ}$C. The Mann-Whitney u test gave no significant difference at the 95% confidence level between the two distributions. Infection at $13^{\circ}$C began earlier after temperature shock with a marked initial fungus attack on day five. Although not statistically significant, the total number of infected fish was slightly higher for both temperatures than that for experiments using a gradual rather than sudden temperature increase.

Figures 18 and 19 give results of experiments V and VI in which a cold temperature treatment was included. A summary of infection times (Figure 19) demonstrates that while fungus infection did occur at $3.5 - 4.5^{\circ}$C, it was both lower in frequency and later in development than at the $13^{\circ}$ or $18^{\circ}$C.

Figure:

    (15)  Effect of warm temperature shock on infection of

           coho with <u>Saprolegnia</u> <u>diclina</u> during

           16 day periods.

FIGURE 15

Fish healthy throughout.

Fish died from fungus infection.

Fish died from causes other than fungus.
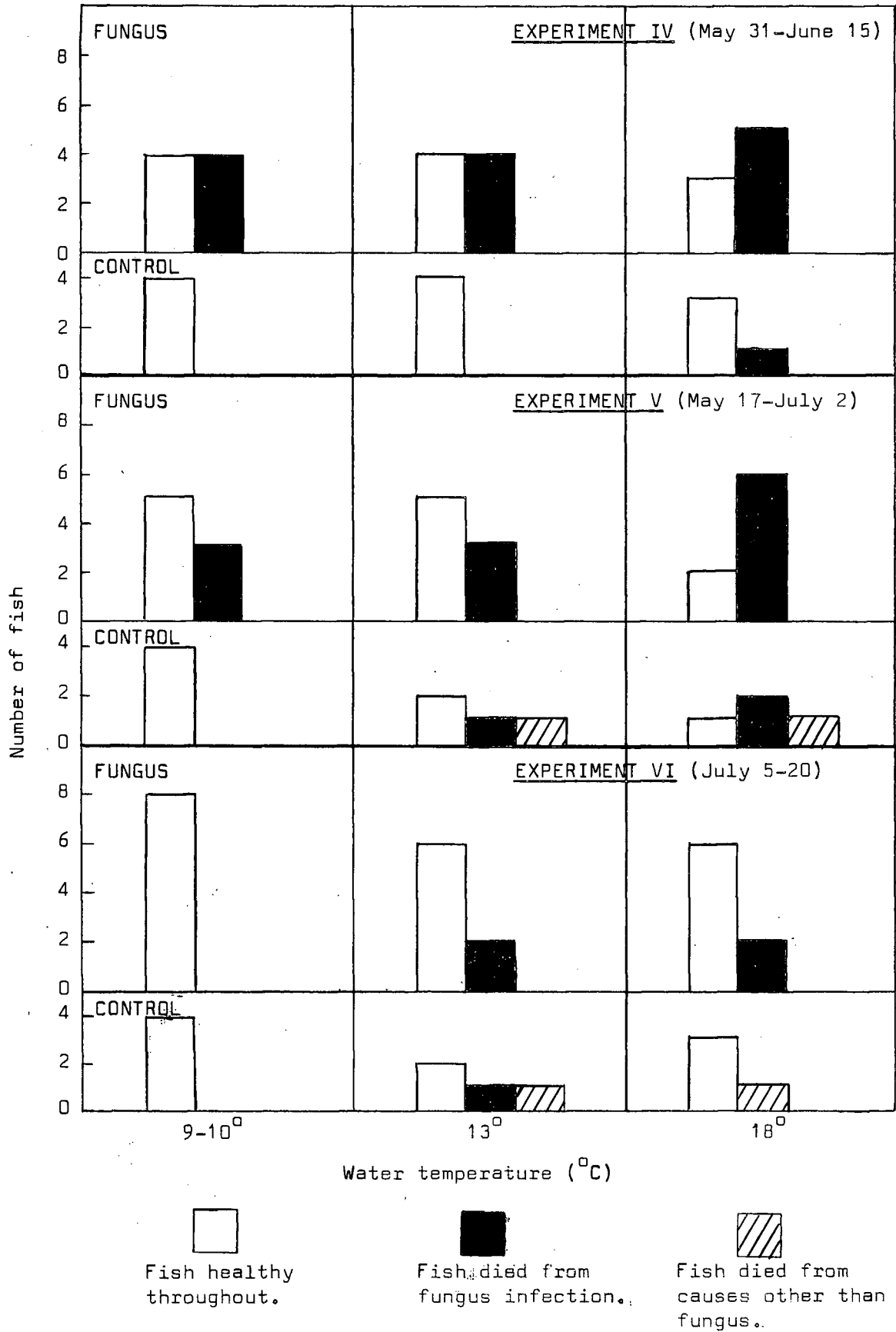
Figure:

    (16)  Effect of warm temperature shock on infection of coho

          with <u>Saprolegnia</u> <u>diclina</u>. Chart summarizes

          results from experiments IV, V, and VI.

FIGURE 16



FUNGUS

CONTROL

Number of fish

Water temperature (°C)

9-10°    13°    18°

☐ Fish healthy throughout.

■ Fish died from fungus infection.

▨ Fish died from causes other than fungus.

Figure:

(17)    Effect of warm temperature shock on time of infection

of coho with <u>Saprolegnia</u> <u>diclina</u> during

16 day period.

FIGURE 17

Day fungus appeared on fish.

Figure:

(18) Effect of warm and cold temperature shock on infection
of coho with <u>Saprolegnia</u> <u>diclina</u> during 16 day
periods. Chart summarizes results from
experiments V and VI.

FIGURE 18

Fish healthy throughout.

Fish died from fungus infection.

Fish died from causes other than fungus.

Figure:

 (19) Effect of warm and cold temperature shock on time

    of infection of coho with *Saprolegnia* *diclina*
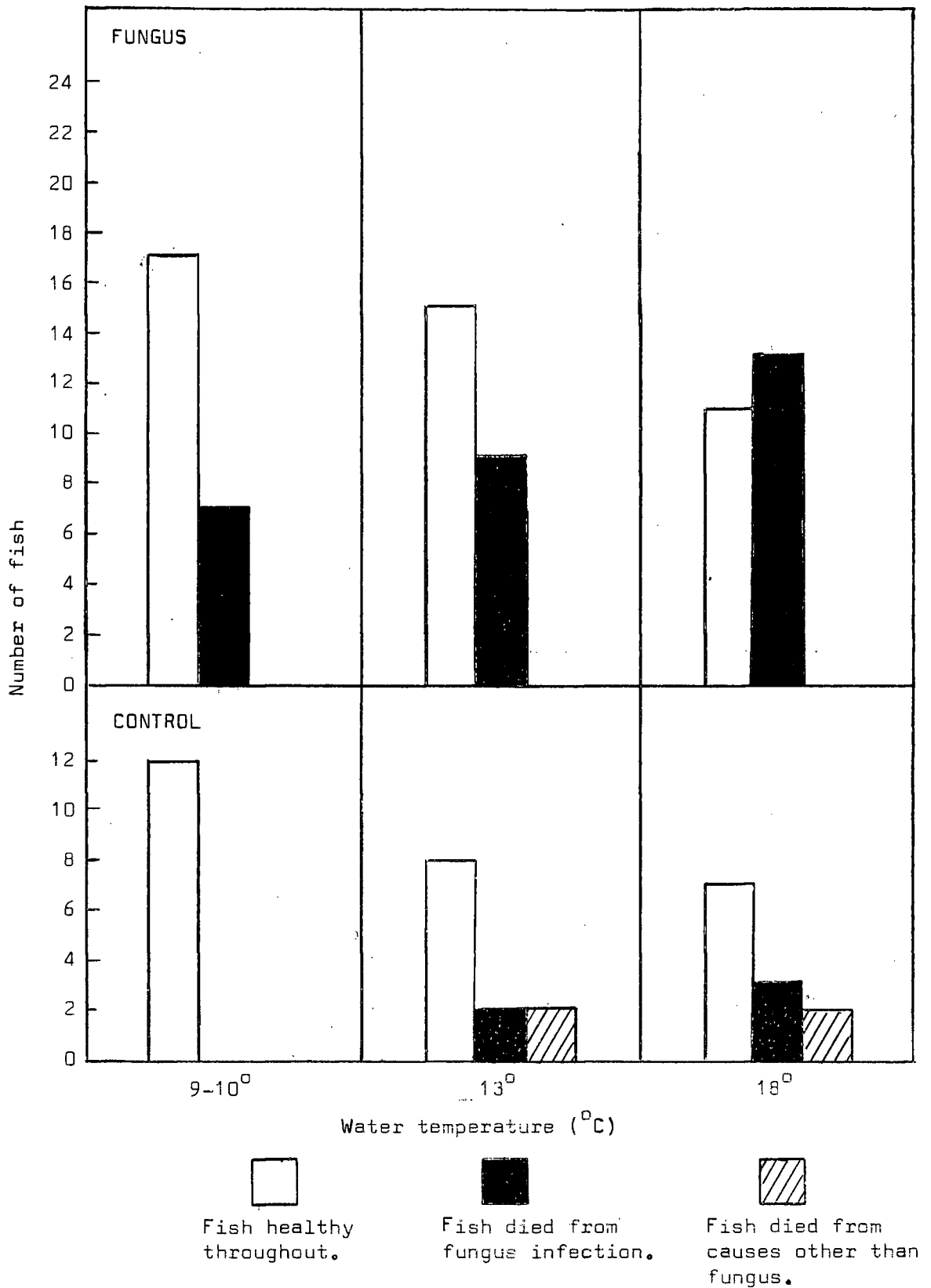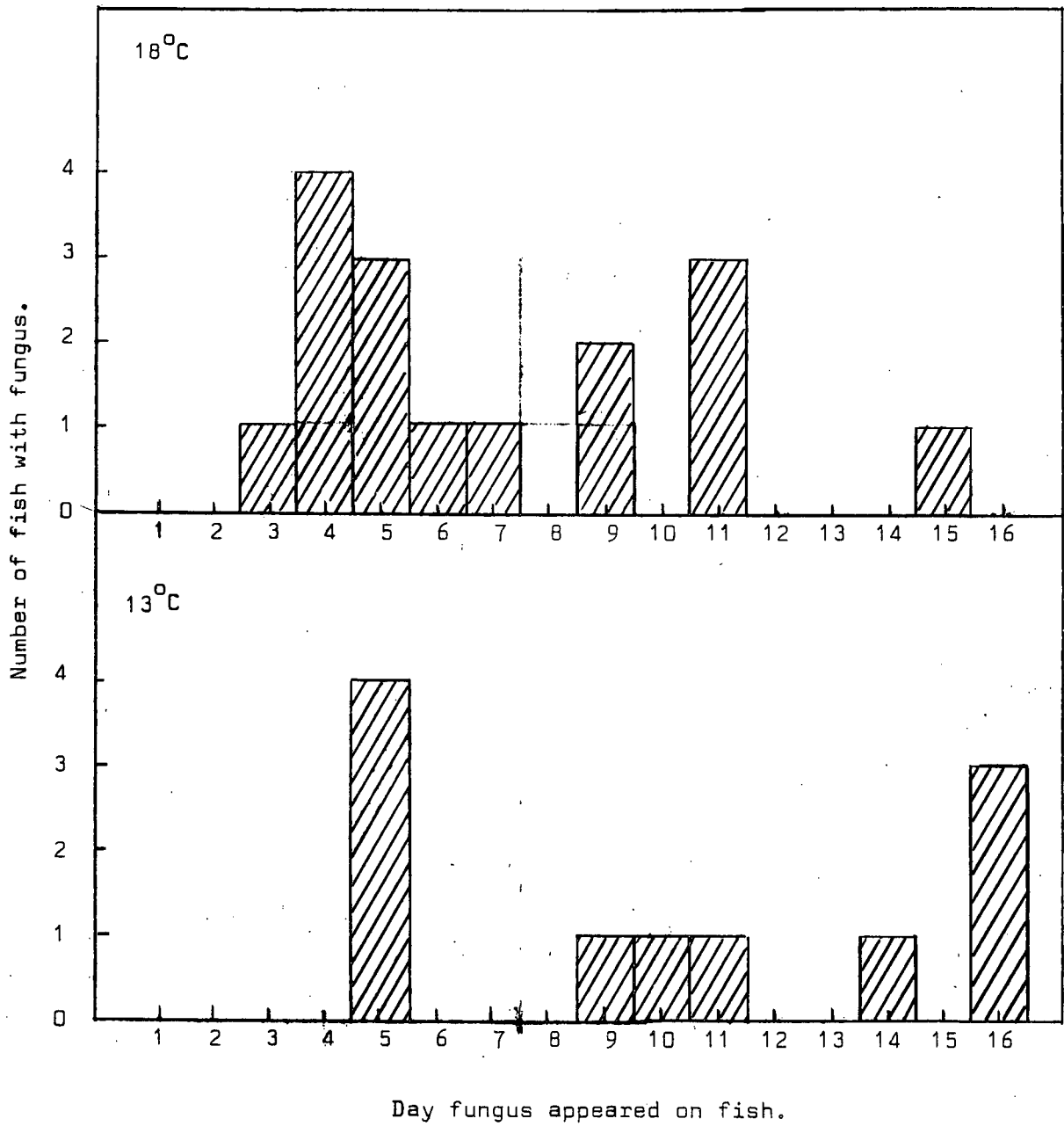
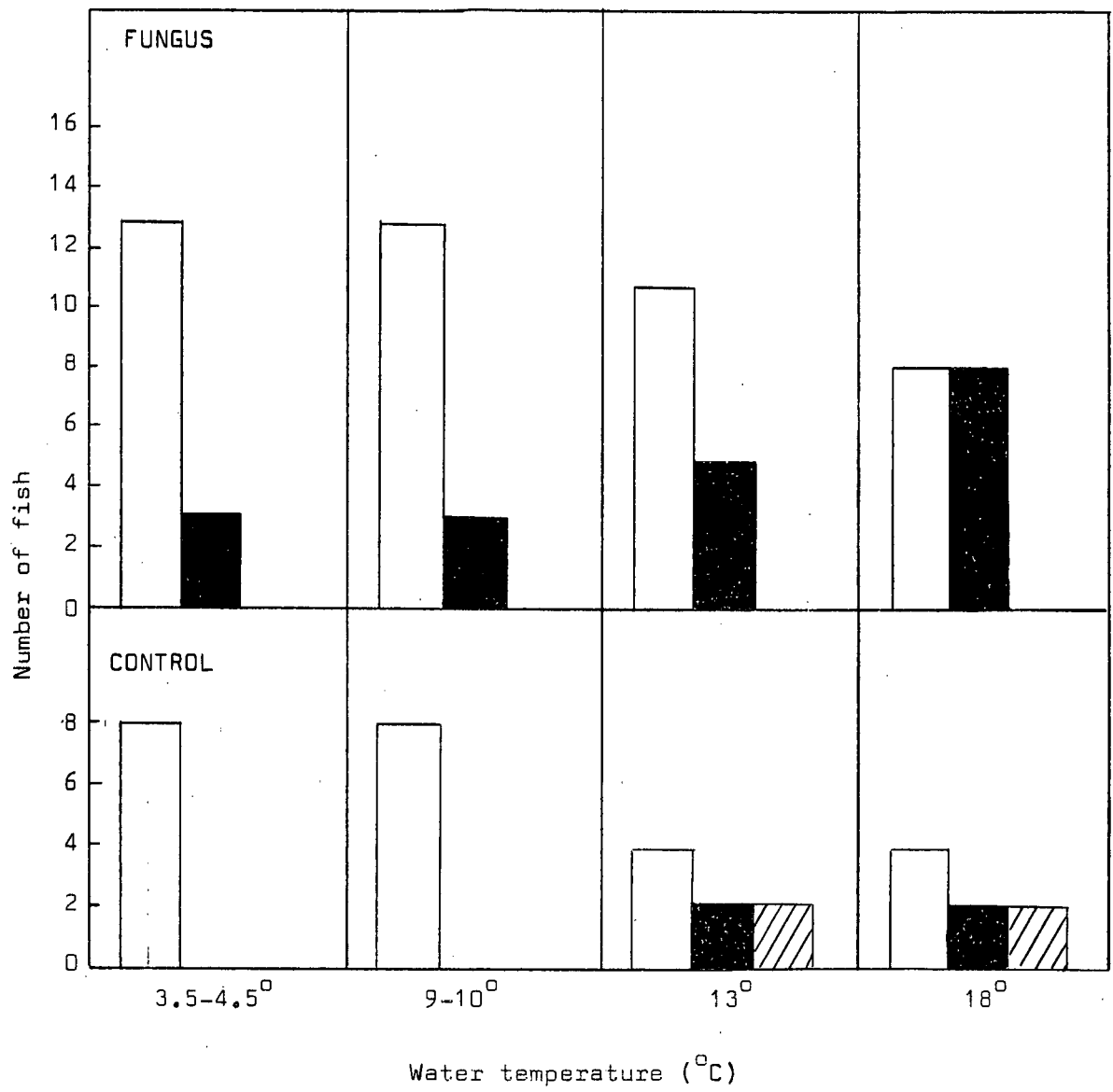    during 16 day periods.

FIGURE 19
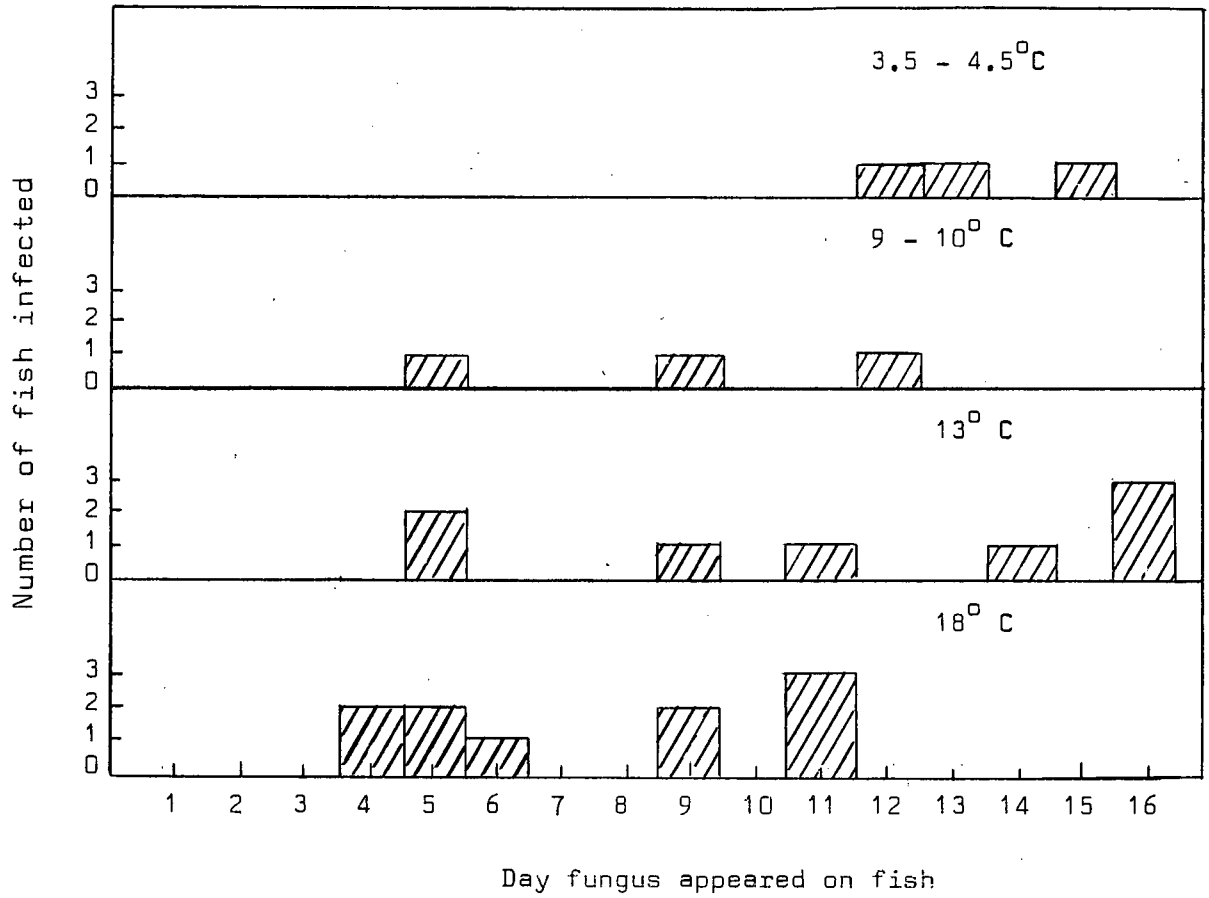
## Observations on fungus development and host response:

Close observation of fish during all experiments revealed striking variations in patterns of fungus development and host response.

Fish response ranged from nervous darting about to apparent indifference to the presence of fungus. Fungus infection was at times preceded by a general sluggishness of the hosts, such fish swimming close to the water surface or lying still on the bottom of the aquarium. At other times, no abnormal symptoms or patterns of behaviour preceded infection. Fungus always began as a small white tuft of hyphae protruding from fish tissues. No ulcerative sores or other eruptions preceded fungus infection. At no time were any fish observed rubbing infected areas against the aquarium sides. As the fungus advanced, infected tissues became progressively paralyzed and fish movement was seriously hampered. Fish eventually lay motionless on the surface or bottom of the aquarium and died shortly thereafter.

The site of infection was extremely variable. The initial infection was often on or at the base of fins, especially the dorsal, caudal, and pectoral fins. Data on infection sites are summarized in Figures 20 and 21. Analysis using a Chi-square test demonstrated non-randomness at the 99% confidence level in the areas of body surface first attacked by fungus. For over 50% of the 76 fish examined, fungus began on area IV, the region of the caudal peduncle and caudal fin. Little difference existed in the frequency of infection initiating on the fins or directly on the body tissues. Of the 42 records of body infections, over 50% began on the caudal peduncle. Only four infections initiated on the gills. Although

Figure:

(20) Infection site frequencies based on observations made

on 76 coho infected with <u>Saprolegnia</u> <u>diclina</u>.

FIGURE 20
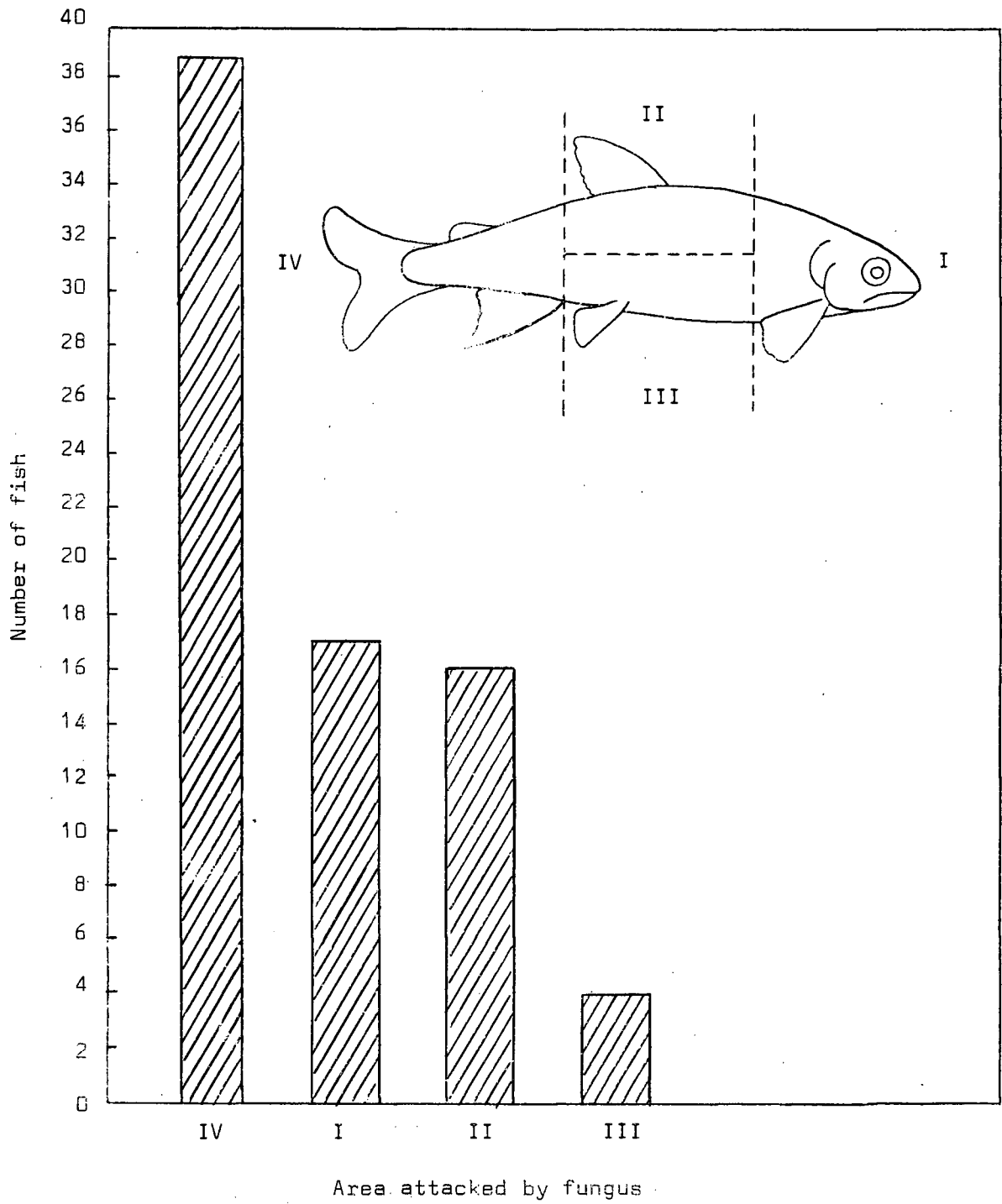
Figure:

    (21)   Summary of specific infection sites based on
            79 observations of initial infections of
            coho with <u>Saprolegnia</u> <u>diclina</u>.
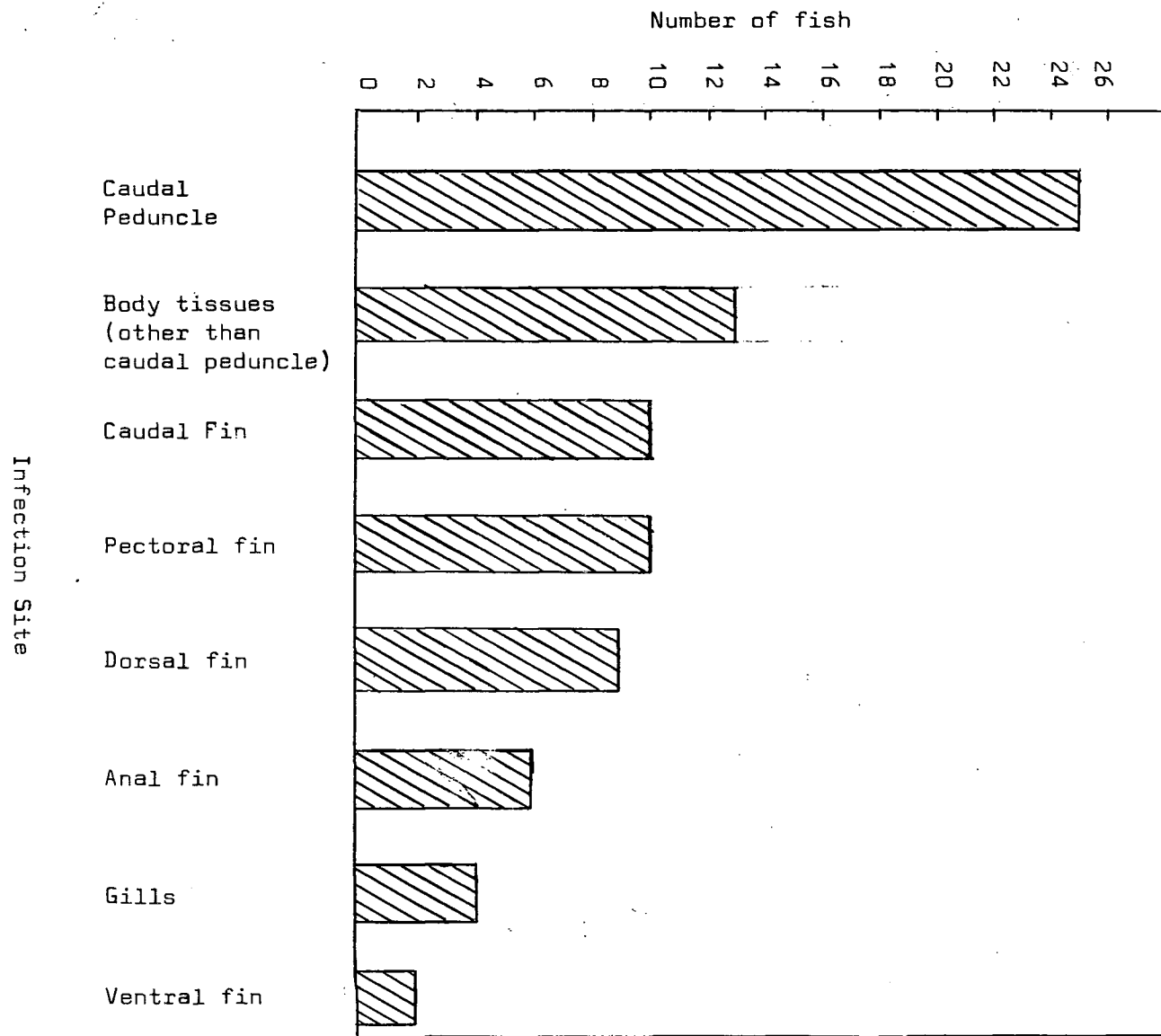
FIGURE 21

rare, such gill infections developed rapidly and were extremely destructive, killing the fish in a few hours. Similar infections often began at the base of pectoral fins from which they progressed quickly to gill tissues.

Once established the fungus spread rapidly over body tissues, killing fish from 12 to 96 hours after its first appearance.

The pattern of fungus growth varied as much as the site of infection. Tissues beneath fungal mats appeared white. Close examination revealed loss of pigment in tissues from which hyphae protruded. Sometimes these unpigmented areas extended 1 or 2 mm beyond the leading edge of the fungus forming concentric ring patterns. Within hours, hyphae erupted from these unpigmented tissues and a second unpigmented band formed outside the first. In another frequent pattern of infection, surrounding tissues showed no such initial response. Fungal hyphae formed a compact white mat closely flanked by normally pigmented fish tissues.

The extent of superficial fungal growth at the time of death varied from a small tuft protruding from one operculum to large fungus patches covering over two-thirds of the body surface. Death never occurred as a result of infection on the fins alone; some body tissues were always infected. Plate 4 shows extent of fungus infection on several fish photographed minutes after death.

In several aquaria, a pattern of aggression developed among the four fish with the largest generally acting as the aggressor. The resulting fin and tail nipping sometimes left smaller fish with ragged fins, especially the caudal and pectoral fins. Although these areas were at times attacked by fungus, they were just as often free of fungus infection. The

PLATE 4

Coho fingerlings infected with Saprolegnia diclina. Arrows indicate extent of external fungus development at time of fish death.

Figure:

(22) Dorsal infection x 2 1/2.

(23) Dorsal infection extending to caudal and ventral

regions. Note mechanically damaged caudal

fin without fungus infection. X 2 1/2

(24) Ventral infection with origin at base of anal fin. x 2 1/2

(25) Infection with origin at the base of caudal and

dorsal fins. x 6

PLATE 4
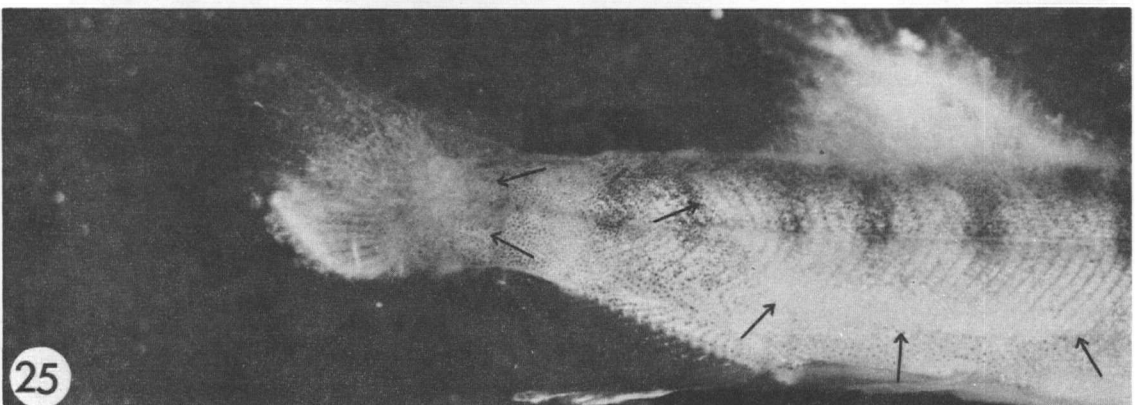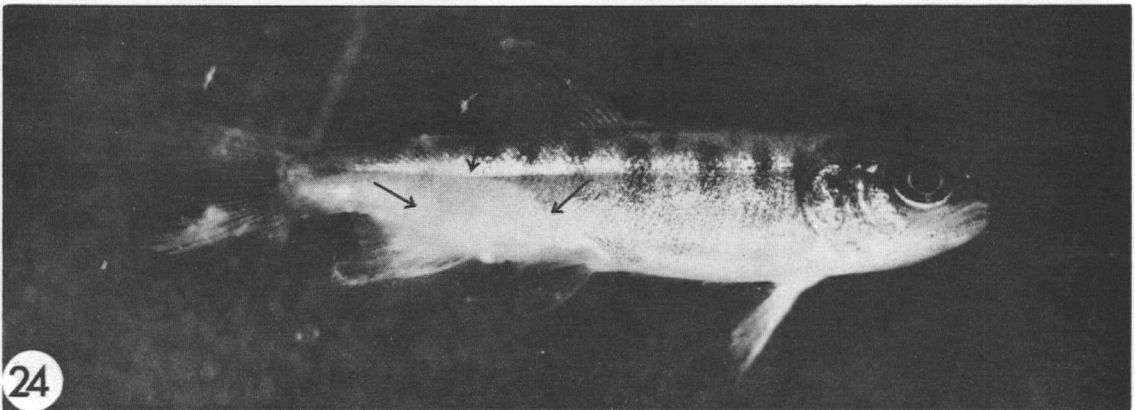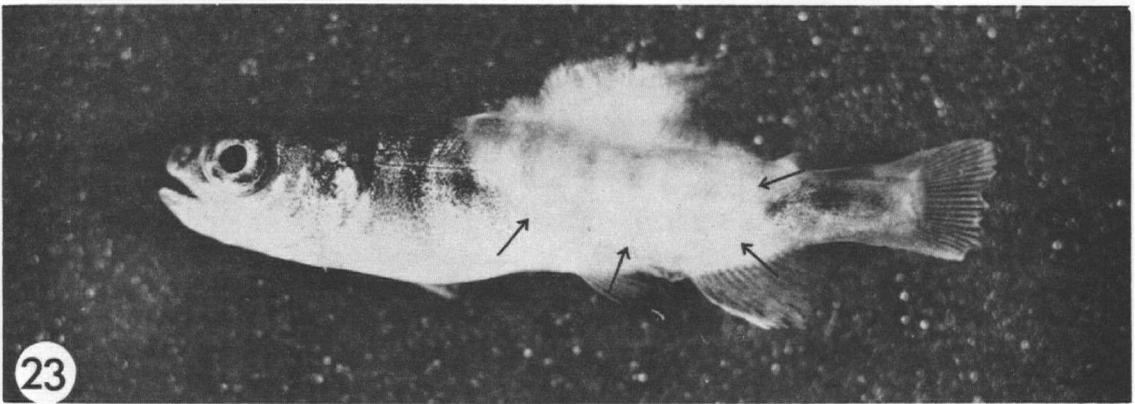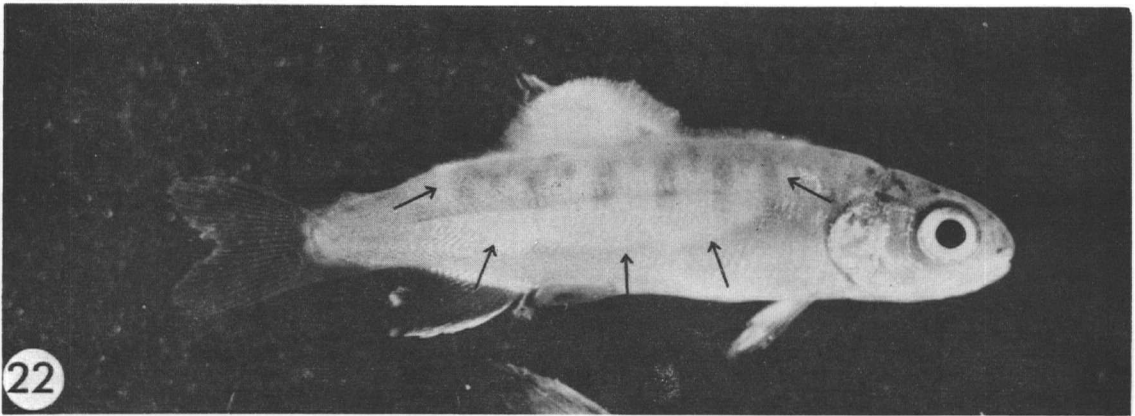
largest and most aggressive fish seldom contracted fungus.

A few fish in both control and experimental tanks contracted an in-fection, probably bacterial, which caused tissue damage of the caudal peduncle. For the few fish involved, such regions were common sites of fungus infection.

Rough post-mortem examinations of all fish -- infected and uninfected -- for internal fungus infections or for parasites other than fungi and bacteria were all negative.

## Histological Studies

Of the three staining techniques used, only the periodic acid-Schiff method gave differential staining. With this method, fungal hyphae appeared light purple to pink against turquoise staining epidermis and musculature and purple staining dermis and connective tissues. Sections through normal and infected tissues are shown in Plates 5 and 6. Internal fungus infections were concentrated in tissues immediately beneath super-ficially infected areas. Fungus was heaviest in the epidermis where, at times, a mesh of branching hyphae completely replaced the layer of epiderm-al cells. Whereas in some sections hyphae were found in the epidermis only, in others they were observed in deeper tissues. Such hyphae penetra-ted thick, dermal tissues to ramify underlying musculature. Although fewer in number than epidermal hyphae, these hyphae were equal in diameter $(5-8\mu)$. Hyphae rarely penetrated more than 2 or 3 mm into underlying muscle tissues. In a few sections, however, hyphae extended through dorsal musculature into the cavity of the vertebral column. Epidermal and dermal infections re-sulted in distinct breakdown of host cells. Muscle infections, however,

PLATE 5

Sections of coho to compare healthy tissues with tissues
infected with <u>Saprolegnia diclina</u>

Figure:

(26)  Section through healthy tissue.

D = dermis; E = epidermis; M = muscle tissue.

x 250

(27)  Section through tissue infected with <u>Saprolegnia</u>

<u>diclina</u>.

D = dermis; E = fragmented epidermis;

M = muscle tissue; F = fungal hypha.  x 300.

PLATE 5
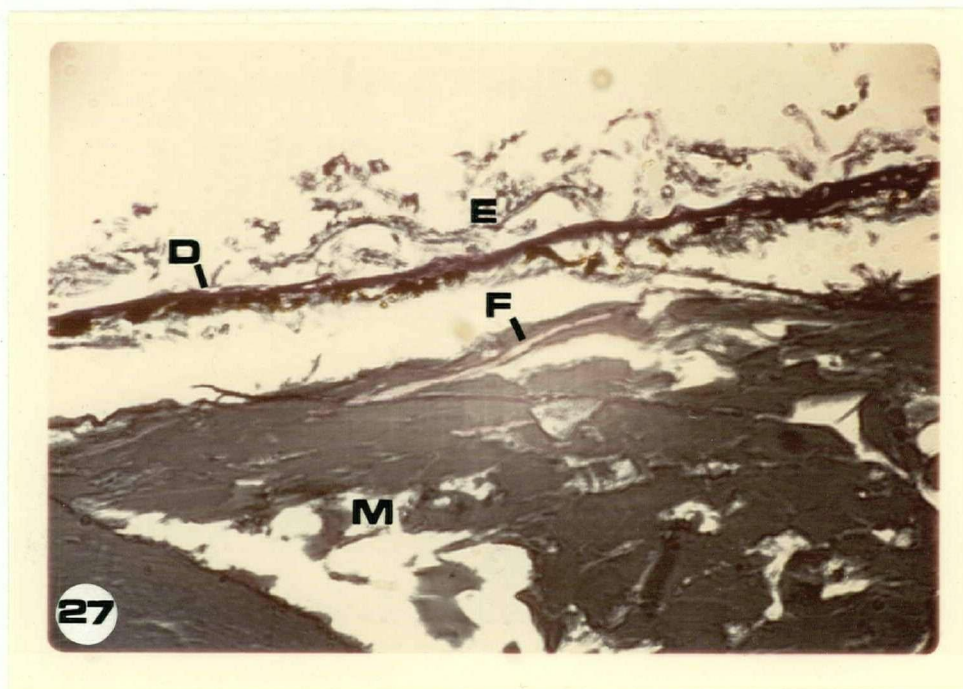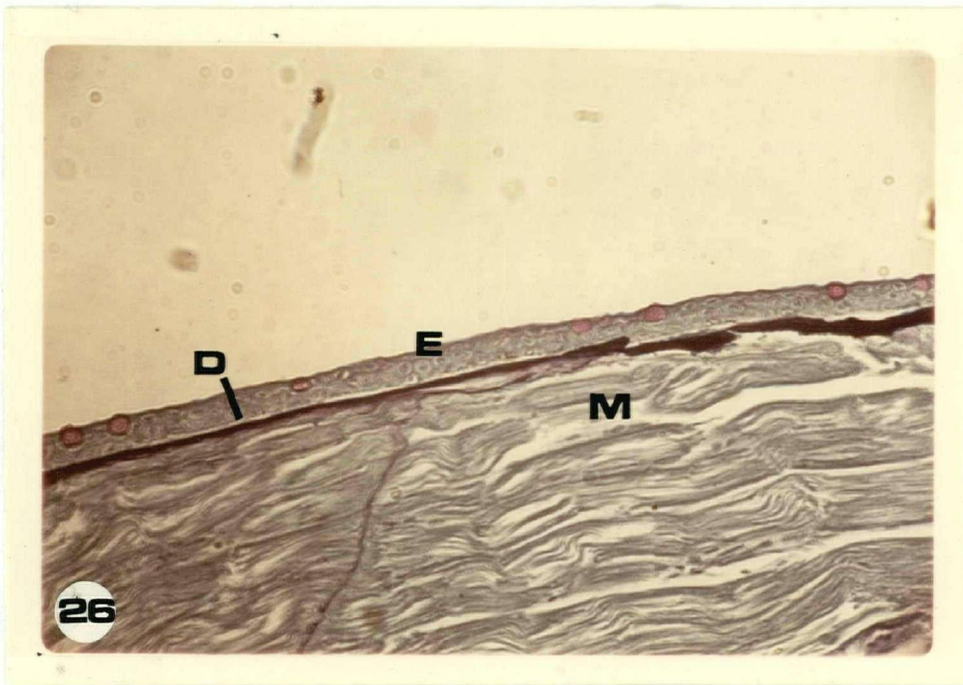
PLATE 6

Sections through fish tissue infected with <u>Saprolegnia</u> <u>diclina</u>

Figure:

 (28) Heavy epidermal and dermal infection.

     D = dermis; E = fragmented epidermis;

     F = fungal hypha; M = muscle tissue. x 200


 (29) Deep infection showing fungal hyphae penetrating well

     into muscle tissues; epidermis completely destroyed.

     D = dermis; F = fungal hypha; M = muscle tissue. x 100

PLATE 6
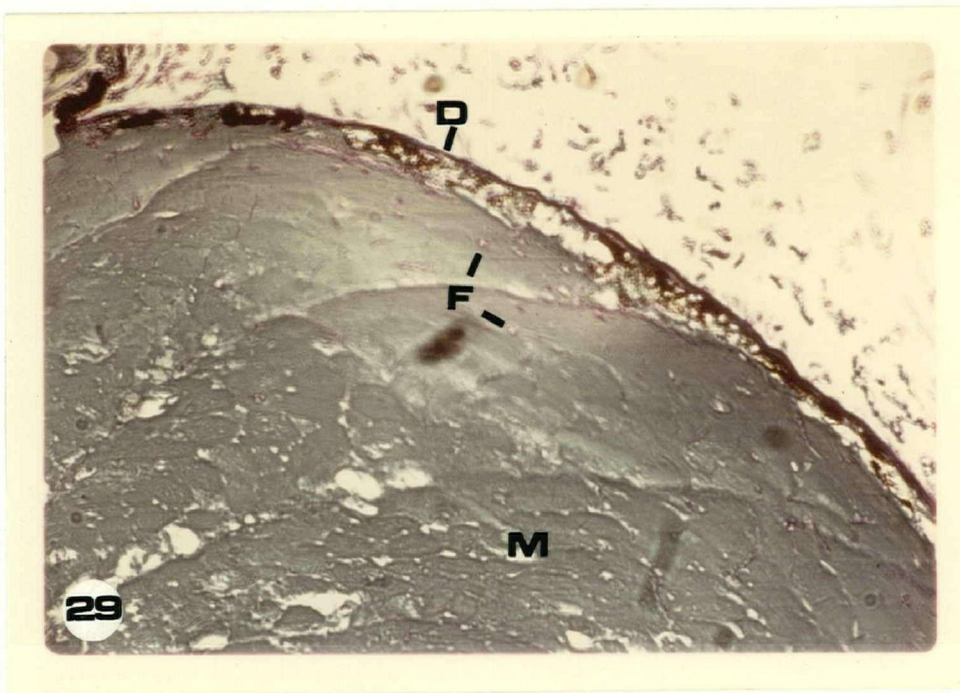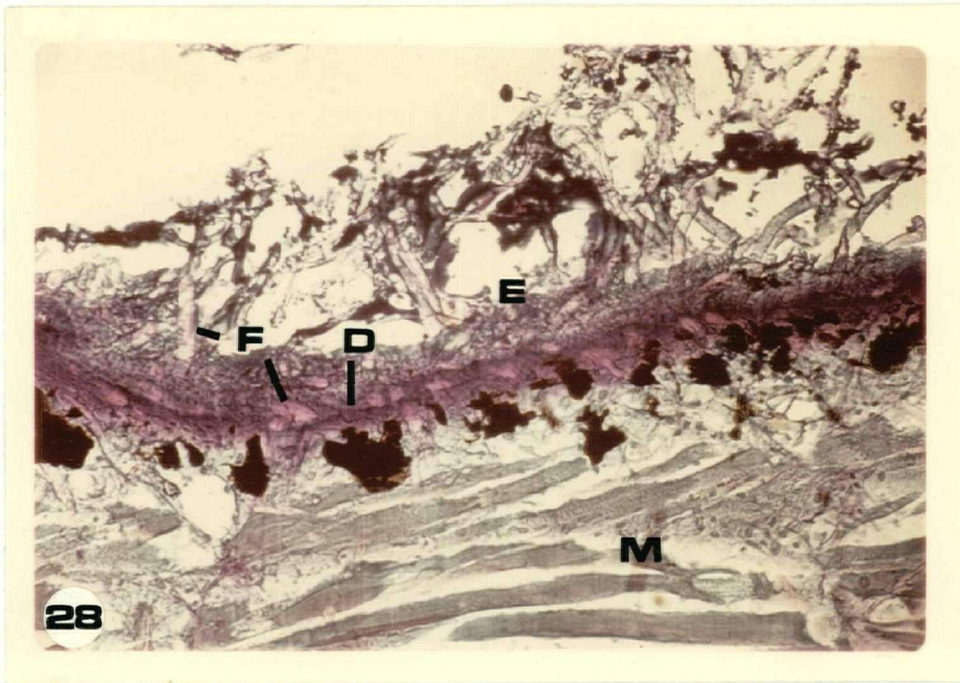
produced little tissue destruction beyond a narrow zone immediately adjacent to the dermis. Except for a small channel created by the hyphal filament, such tissues often appeared normal. In no sections were fungal hyphae observed within internal organs.

## DISCUSSION

Our understanding of Saprolegniaceous infections among fish -- their biology, effects, and overall significance -- is very inadequate. An evaluation of previous studies is complicated by many factors. One of the most serious difficulties arises from the use of non-standardized culture and identification procedures. Many references to pure-culture techniques are found in the literature (Raper, 1937; Tiffney, 1939 b; Johnson, 1956; Seymour, 1966). Although the methods used were reported successful, similar techniques used in this study proved only partially satisfactory. Results indicate that a completely "pure", bacteria-free culture of a parasitic water moold is a rare phenomenon! The theory that bacteria will not move along hyphae which penetrate a solid, agar medium does not appear valid for all cases. This situation is perhaps explained by the fact that many of the aquatic bacteria are very motile and tolerant of near anaerobic conditions. Furthermore, both bacteria and fungi are known to produce strong enzymes (Stanier, et al., 1963; Lilly and Barnett, 1951), many of which appear capable of liquifying an agar gel. The presence of thin films of liquid agar surrounding hyphae cut out of solid cultures during this study may be the result of digestive enzymes produced by the growing hyphal tip. Viable bacteria moved as freely through this zone as through a liquid nutrient medium.

Perhaps the reason that previous investigators were satisfied with usual pure-culture techniques is because of the fact that contaminated cultures often appear pure. Only close microscopic examination or growth on a rich, nutrient medium reveals presence of bacterial contaminants.

During this study, cultures which initially gave clear solutions in liquid medium often turned cloudy after a period of time. Such delayed evidence of contamination may have resulted from the presence of resistant bacterial spore stages. The most effective pure-culture technique during this study involved repeated transfer of cultures using combinations of known procedures together with periodic transfer to a nutrient-rich liquid medium as a test for contamination.

Results obtained during these culture studies justify some comment on the use of ultra-violet irradiated media. Whereas many cultures during this study grew successfully on such media, some did not remain viable. It is felt that, even though irradiation was not applied directly to the fungi, the resulting products were capable of preventing fungal as well as bacterial growth. The technique was eventually abandoned owing to the fear of introducing mutant strains of fungi. Unless further investigations are made on the effect of such media on the fungi, this technique should probably be regarded with some suspicion.

The problems complicating identification of these parasitic fungi are innumerable. Countless studies, both in the past and more recently, have done little to clarify the confusion. Perhaps the basis of the trouble stems from the characteristics used to distinguish genera and species in the Saprolegniaceae. Hyphal diameter and growth habit, measurements and structure of sexual structures, measurements and development of asexual structures, spore production and diameter -- all are used in the separation of species. Several, as has been shown by Salvin (1941, 1942), are subject to a high degree of variation within a species or genus. In

many keys, e.g., Coker, 1923, extreme measurements for species often over-
lap, hemp seeds form the standard medium, and identification often necessi-
tates rough estimates. Seymour, in a recent monograph of the genus
Saprolegnia (1966), attempted to revise the genus on the basis of growth
on a chemically-defined standard medium. Although Seymour's approach to
the problem has long been overdue, his choice of medium may not be entire-
ly suitable. Several attempts to grow fungi isolated in this study on
Seymour's MSPS medium were unsuccessful. No logical explanation has been
found to account for this failure unless a faulty or contaminated chemical
was used.

Since the most commonly reported fungus parasite of fish is S.
parasitica, a closer look at the taxonomic position of this species seems
necessary. S. parasitica was originally defined purely on the basis of
presence on fish tissues and lack of sexual reproductive structures
(Coker, 1923). Most other characteristics were similar to many other
species of Saprolegnia. When sexual reproduction was observed (Kanouse,
1932), the fungus was redescribed and retained as a distinct species. The
main distinguishing points of Kanouse's description are given in Table 3.
Only three characteristics differentiate her species from certain other
Saprolegnia species, as, for instance, S. diclina. These are growth on
fish, reduced ability to produce sexual structures, and presence of sub-
centric oospores. In order to demonstrate some of the problems inherent
in Kanouse's description of the species it is necessary to consider all
three characteristics individually.

As has already been demonstrated in Table 1, many species of

TABLE 3: Comparison of the morphology of _Saprolegnia parasitica_ Coker and _S. diclina_ Humphrey

| Species | Zoo-spore Diameter | Oogonium shape | Oogonium size | Antheridial type | Oospore type | Oospore Diameter | # Oospore per Oogonium | Reference or Source |
|---|---|---|---|---|---|---|---|---|
| S. parasitica | 9-11.5 | -- | -- | -- | -- | -- | -- | Coker, 1923 |
| S. parasitica | 12 | pyriform, clavate, sub-spherical to spherical | 65-135 x 60-95 | diclinous, few androgynous | sub-centric | 18-22 | 3-25 | Kanouse, 1932 |
| S. parasitica | 9-11 | clavate to pyriform or irregular | 75-850 x 20-80 | diclinous | sub-centric | 16-28 | 2-40 | Seymour, 1966 |
| S. diclina | | spherical, oval or pyriform | d 35-100 | diclinous | centric | 20-26 | 1-20 | Coker, 1937 |
| S. diclina | 10-12 | spherical or pyriform | 32-110 x 52-65 | diclinous | centric, occasionally sub-centric | 12-36 | 1-28 | Seymour, 1966 |
| S. diclina | 10-13 | spherical to oblong | d 45-80 | diclinous | sub-centric | 15-28 | 1-20 | Robertson Creek, 1966 |

Saprolegnia as well as several other genera of the Saprolegniaceae have
been reported as fish parasites. S. parasitica itself is not limited to
growth on fish tissue and grows freely in culture as a saprophyte on
various seeds and other media. Thus, growth habit in no way distinguishes
this species from certain other members of the genus.

The second distinguishing characteristic essentially involves a
degree of ability to produce oogonia and antheridia. At present little
is known of the factors inducing sexual reproduction in this fungus. It
has been suggested (Kanouse, 1932; Rucker, 1944) that a reduction of food
supply induces sexual reproduction. This principle, used for other species
of Saprolegniaceae by Klebs (1899), Kauffman (1908), and Pieters (1915),
has been used to explain the failure of oogonia and antheridia to develop
on fish tissues. Although the principle may apply for S. parasitica, it
does not apply for all other parasitic fish molds. The fact that, during
this study, a small patch of fungus producing sexual structures was
observed among hyphae on a heavily infected fish suggests more thorough
examination of tissues is necessary before concluding sexual reproduction
does not occur on fish. The same principle has been used by Kanouse to
explain the development in culture of oogonia and antheridia on S.
parasitica grown in dilute peptone and maltose or peptone and leucine
solutions. No such structures, however, developed during the present
study when the C.B.S. culture was grown in a duplicate series of Kanouse's
media. Sexual reproduction was recently observed in three cultures grown
in liquid MSM medium containing a few drops of corn oil. The possibility
of a sexual stimulant in corn oil was suspected. However, since a repeti-
tion of the treatment failed to duplicate results, it was felt some other

factor, e.g., contamination of glassware, induced sexual reproduction.
Occasional reports of oogonia and antheridia produced by S. parasitica
are found in the literature (Tiffney, 1939 a; Rucker, 1944; Scott, 1964;
Seymour, 1966). A brief comparison of these descriptions is given in
Table 3. In all cases, the most significant, common factor was the long
period of time prior to sexual reproduction. Scott reports "...extreme
variability in the time required for formation of sexual organs" with a
minimum time of over four weeks after isolation and a maximum of well over
a year. The problem of delayed sexual reproduction is not limited to
S. parasitica. Tiffney (1939 b) reports a culture of S. ferax which did
not reveal its identity for some time after isolation. On the basis of
similar asexual structures, it had been tentatively assigned to S.
parasitica. In the present study several cultures of S. diclina were slow
to produce oogonia and antheridia. Until more is known of the factors
inducing sexual reproduction in S. parasitica and other species of the
genus, accurate identification will continue to be seriously hindered.

The third important feature of S. parasitica -- production of
subcentric oospores -- may also have limitations as a distinguishing
characteristic. The fungus, S. diclina, has also been reported to occa-
sionally produce subcentric oospores (Seymour, 1966). Isolates of
S. diclina used in the present study consistently produced such oospores.
In these isolates, two forms of subcentric oospores were observed --
oospores with one layer of oil droplets on one side of the central ooplasm
and several on the other, and oospores with two to three layers on one
side and up to five on the other. The first type was consistent with
Johnson's (1956) definition of subcentric; the second, as yet not

described in the literature, could represent an intermediate form between centric and subcentric. That oospore type within a single species can be so unstable suggests that it is perhaps a poor taxonomic criterion. Further investigation of potential variability of oospore type in S. diclina, S. parasitica, and perhaps in other species of the genus is necessary before using it as a major distinguishing characteristic.

Kanouse's early description emphasizes another major problem in identification of S. parasitica -- the considerable capacity for variation within a single isolate. She found that "...on solid media, nearly all oogonia were spherical and antheridial filaments were androgynous. Many oogonia developed without antheridia. In liquid environments, oogonia were spherical, subspherical, to pyriform or clavate and many were inter-calary. The antheridial branches were always diclinous. Very few oogonia developed parthenogenetically." (Kanouse, 1932). Tiffney (1939 b) in an extensive study of the morphology of S. parasitica isolated eight separate strains on the basis of variation in measurements of asexual reproductive structures alone. A considerable overlap of measurements, however, re-sulted in the eight strains forming a continuous series. Table 3 compares pertinent characteristics of isolates of S. parasitica described in the literature. Observations on sexual structures of S. parasitica made during the present study were incomplete owing to the small number of oogonia observed and failure of oospores to fully mature.

For comparison, descriptions of S. diclina from this study and from several other sources are also given. Both the high degree of variability recognized within the species S. parasitica and the basic similarity of

sexual reproductive structures with those of S. diclina became apparent.
It is interesting to note that Scott (1964) reported that 14 isolates of
S. parasitica differed little from isolates of S. diclina except in the
complete absence of oogonial pitting.  Since S. diclina is itself "...with-
out pitting except where antheridia touch" (Coker, 1923), the difference
becomes less and less clear.

The above comparison has not been made to suggest S. parasitica is
synonymous with S. diclina but rather to emphasize the fact that S.
parasitica is an extremely ill-defined taxon, even on the basis of its
sexual structures.  Criticism in the past has been directed to identifica-
tion on the basis of asexual structures alone.  For this reason Rucker
(1944) referred to S. parasitica as the "waste-basket" for Saprolegnia;
Scott (1964) called it a "catch-all" for non-fruiting, parasitic isolates;
Tiffney (1939 b) spoke of forms of "...heterogeneous origin which, if they
could be induced to fruit, might belong to several different species within
the genus, Saprolegnia."  The present author would also question identifi-
cation on the basis of sexual organs since their particular morphology
does not appear to differ significantly from that of certain other members
of the genus.  The presence of physiologic strains within species of
Saprolegnia is suggested as a possible explanation for the variable capacity
for sexual reproduction between isolates and between certain species.
Furthermore, the possibility exists that certain of these strains differ
in their requirements for sexual reproduction.  Some of these fungi may
have very narrow limits in the various environmental and nutritional
factors required for the production and maturation of sexual organs.

Because of the insecure status of the taxon, $\underline{S}$. parasitica was considered a poor organism to use during experimental studies. For this reason $\underline{S}$. diclina was used in all infection studies. The isolate used resembled Coker's $\underline{S}$. diclina Humphrey for all but one characteristic -- production of subcentric oospores. As has already been pointed out, $\underline{S}$. diclina does occasionally produce typical subcentric oospores (Seymour, 1966). The stability of oospore type has also been questioned. For these reasons the presence of subcentric oospores rather than centric oospores was not considered a valid criterion for placing the isolate in a new species. For the purpose of this study, therefore, the fungus has been referred to as $\underline{S}$. diclina Humphrey.

The unusual morphology of $\underline{S}$. diclina observed on fish tissues is perhaps an adaptive mechanism for spore dispersal. In fast-flowing, often turbulent streams, tiny zoospores must often be destroyed or lost before contacting a suitable substrate for germination. The production of aplanoid sporangia allows whole sporangia to act as dispersal mechanisms. These structures could feasibly be carried long distances before spores are released thus increasing spore distribution through time and space.

The question "Is the fungus a primary or secondary parasite?" has been repeatedly asked but never completely answered in the literature. The fact that infection requires some predisposing condition is generally accepted. Several suggested conditions have already been outlined. Results from the present infection studies indicate infection is definitely correlated with some such predisposing condition. Certain facts regarding this condition have also been elucidated. Mechanical injury, although an important factor, is not the main condition leading to infection.

Treatment of fish both prior to and during infection studies attempted to minimize mechanical injury. Fish nevertheless were susceptible to fungus attack. It was also noticed that occasional injuries resulting from aggressive fin and tail nipping were not necessarily sites of infection. Although exposed to a continual supply of fungus spores, these fish often remained free of fungus infection throughout the 16 or 18 day experiments. The fact that injury alone may not be enough to predispose fish to fungus attack was suggested by Rucker (1944). Present data appear to confirm his opinion.

On the whole, the possibility of temperature as a predisposing agent has met with little support (e.g., Murray, 1885; Rucker, 1944). Rucker states that in early attempts to inoculate trout and salmon fingerlings with S. parasitica "...retention of fish at temperatures approaching the maximum for fish did not cause fungus to develop." He concluded that fungus disease, among the salmon and trout examined, followed a primary bacterial invader, Chondrococcus columnaris. The bacterial infection was attributed in part to high summer temperatures.

Results from the present study do not concur with Rucker's theory that a primary bacterial infection is the necessary predisposing agent. Although such infections are undoubtedly vulnerable sites for secondary fungus infections (unpublished report, Wood, 1965), they do not appear to be an absolute requirement for fungus infection. Careful observations during the present studies indicated that at higher temperatures the fungus infected fish regardless of the presence or absence of obvious bacterial infections. At lower temperatures fish were almost completely resistant

to fungus attack. As temperature increased, susceptibility to fungus infection also increased. The time required for fungus to develop, on the other hand, was decreased.

The temperatures used during experiments I to VI were chosen on the basis of results from trial experiments and field observations at Robertson Creek. Both indicated little infection occurred at $8^\circ$C while relatively serious infection began at $13^\circ$C or above. All temperatures used were within the growth range of $\underline{S}$. $\underline{parasitica}$ (Duff, 1929; Powell, 1966) as well as that of other species of $\underline{Saprolegnia}$ (Pieters, 1915; Cotner, 1930; Perrott, 1960).

Rucker's failure to obtain infection at higher temperatures is puzzling on the basis of present results. However, it is possible that the fungus he used had a different optimum temperature for infection or that it belonged to a less virulent strain than the one used in the present study.

The fact that fungus infection became relatively serious at $13^\circ$C seems to coincide with reports of serious bacterial and fungal infections at Robertson Creek at temperatures above $13^\circ$C. Wood (1965), in a recent study of pre-spawning mortalities in the Fraser River, suggests correlation with serious $\underline{C}$. $\underline{columnaris}$ infections and spawning ground temperatures in excess of $13^\circ$C. So many reports of high mortalities, due to bacteria, fungi, or both, suggest some significance should be attached to temperatures close to $13^\circ$C. Recently, pathologists have begun to consider with some interest the fact that few diseases, except those having reached epidemic status, attack healthy individuals in their normal environment. Certain environmental changes will often upset the physiology of an organism with

the result that certain natural resistance mechanisms are broken down. It is suggested that, at temperatures at or above 13°C, salmon may lose much of their resistance to bacterial and to fungal infections.

Whether or not Saprolegniaceous fungi are serious disease organisms among salmon fingerlings in nature, these facts remain clear: S. diclina, a common fungus in British Columbia waters, can and does act as a primary invader; fungus infections are generally, if not always, lethal; infection is correlated with higher temperatures; and, finally, the temperatures at which such infections occur in the laboratory are found in certain artificial spawning grounds in British Columbia.

Treatment with sudden temperature shock has not been reported in the literature. Present studies indicated that such treatment, while slightly altering the pattern of infection, did little to change the overall incidence of infection. A comparison of figures 13 and 16 reveals a striking similarity in the results obtained at 13° and at 18°C. The fact that during heat-shock treatment, the 8°C tank reached 10°C during an unexpected heat spell explains the presence of fungus infection in these tanks. Cold temperature shock did not appear to break down resistance to fungus attack. Prolonged cold temperature treatment at 4°C, however, did result in some infection. Further tests are necessary before drawing conclusions on the overall effect of such lower than normal temperatures on fish susceptibility to fungus infection.

The mechanism by which Saprolegniaceous fungi kill fish is still poorly understood. Present studies do little to clarify this problem. Close observations at frequent, sometimes hourly intervals, revealed a

considerable amount of variation in the course of the disease. Whereas
some fish died within hours of the first evidence of fungus, others re-
mained alive for as long as six days. Some died with apparently minor
infections; others with heavy infections covering much of the body surface.
Rucker (1944) reports a similar lack of correlation between extent of body-
surface infection and time of fish death. On the basis of so much variation,
it is difficult to assign death to any one factor. While tissue damage
might account for deaths from heavy infections, it does little to explain
deaths from very light infections extending over but a few square milli-
meters of body surface. Although Rucker (1944) was unable to demonstrate
production of exotoxins, the possibility of toxins cannot be discounted.
The fact that on most fish, the leading edge of visible fungus infection
was often preceded in the present study by loss of dark pigments in adja-
cent tissues, indicates some chemical change probably occurs. This change
is perhaps brought about by substances produced by fungal hyphae as they
penetrate epidermal and dermal tissues prior to eruption.

Results from histological studies indicate that the fungus is con-
centrated in superficial tissues, especially the epidermis. In all heavily
infected regions, the layer of epidermal cells was almost completely re-
placed by fungus hyphae. The dermis layer in young O. kisutch is limited
to tough connective tissues with little spongy tissue. The fungus was
observed penetrating directly through this tough layer and into tissues
below. Whereas hyphae caused serious tissue damage in the epidermis and
dermis, they had little mechanical effect on muscle tissue. Muscle infec-
tions rarely extended more than a millimeter or two from the surface.
Tiffney (1939 a) has suggested superficial mechanical injury results in

ionic upset serious enough to kill the fish.  Here, too, the problem of variation in extent of infection and death of fish presents an argument against the theory of ionic upset as the sole cause of death.

The answer to the above problem, as to the many others surrounding Saprolegniaceous infections of fish and other aquatic vertebrates, may never be found in one, single factor.  The considerable variation in all aspects of the disease -- host susceptibility, fungus morphology, development, and course of infection -- lead one to conclude that the disease requires much further investigation.

SUMMARY

Parasitic Saprolegniaceous fungi were isolated from fish from several localities in British Columbia, obtained in pure culture, and identified. Saprolegnia diclina Humphrey was found in five of the seven localities sampled.

Studies of S. diclina as it appeared on fish tissue revealed morphological differences from the fungus grown in culture. Primary zoospores were retained in the sporangium which itself often acted as a dispersal agent. Sexual reproduction was observed on fish tissue.

The problems in identification of many species of Saprolegnia and the status of the taxon, S. parasitica, have been examined and discussed in relation to observations of British Columbia fungi and references to the literature.

Experimental infection studies on fingerling coho with S. diclina were carried out to determine the effect of temperature on infection rate. At $8^{o}$C no infection occurred. Infection began at $9^{o}$C and increased with a further increase in temperature. Infection occurred earlier at higher temperatures. Fish treated with heat-shock were infected sooner than those treated with a gradual change over the same temperature difference. Cold-shock resulted in some infection. However, as infection was delayed, it could not be correlated directly with shock treatment.

Close observation of infected fish revealed the existence of much variation in the development, course, and extent of the disease.

Histological studies demonstrated hyphal penetration and heavy tissue damage in the epidermis, some in the dermis, and little in the underlying muscle tissues. No hyphal penetration was observed in internal organs.

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APPENDIX

## TABLE 4

### MSM Medium

| | | | | |
|---|---|---|---|---|
| 1. | Buffer: | $KH_2PO_4$ | 1.0 | g/l |
| 2. | Inorganic nutrients: | $CaCl_2.6H_2O$ | 0.005 | " |
| | | $CoCl_2.6H_2O$ | 0.002 | " |
| | | $CuSO_4$ | 0.0002 | " |
| | | $Fe(NO_3)3.9H_2O$ | 0.0002 | " |
| | | $H_3BO_3$ | 0.002 | " |
| | | $MnSO_4.4H_2O$ | 0.0001 | " |
| | | NaCl | 0.01 | " |
| | | $(NH_4)_6Mo_7O_{24}.7H_2O$ | 0.002 | " |
| | | $ZnSO4.7H_2O$ | 0.0002 | " |
| 3. | Organic nutrients: | Casein hydrolysate | 1.5 | " |
| | | Dextrose | 2.0 | " |
| | | Biotin | 0.005 | mg/l |
| | | Inositol | 5.0 | " |
| | | Nicotinuric acid | 1.0 | " |
| | | P-Aminobenzoic acid | 0.1 | " |
| | | Pantothenol | 0.5 | " |
| | | Pyridoxine-HCl | 0.1 | " |
| | | Riboflavin | 0.5 | " |
| | | Thiamine | 0.1 | " |
| 4. | Agar (for solid media only) | | 20.0 | g/l |
| 5. | Distilled carbon-filtered water | | 1.0 | l |

6. Dissolved $KH_2PO_4$, casein hydrolsate, NaCl, and agar in 900 ml water in 1 l. flask with detachable 25 ml dispenser. Using sterile pipettes, added 2 ml stock mineral solution A and 1 ml stock mineral solution B.

7. Dissolved dextrose in 100 ml. water in separate Erhlenmeyer flask.

8. Autoclaved flasks from steps 6 and 7 at 15 lbs for 15 minutes.

9. Added dextrose solution to hot medium.

10. Using sterile pipette, added 2 ml. stock vitamin solution to slightly cooled medium.

## TABLE 5

### Stock Solutions for MSM Medium

Stock Mineral Solution A:

1.   
| | | |
|---|---|---|
| $CuSO_4$ | 0.4398 | g/l |
| $Fe(NO_3)_3.9H_2O$ | 0.7235 | " |
| $MnSO_4.4H_2O$ | 0.203 | " |
| $ZnSO_4.7H_2O$ | 0.4398 | " |

2.   Dissolved ingredients from step 1 in 600 ml. distilled carbon-filtered water.

3.   Added enough concentrated $H_2SO_4$ to yield a clear solution.

4.   Made volume up to 1 liter with water.

5.   Autoclaved at 15 lbs for 15 minutes; stored at $13^oC$.

6.   Used 2 ml. stock solution per liter medium.

Stock Mineral Solution B:

1.   
| | | |
|---|---|---|
| $CaCl_2.2H_2O$ | 0.05 | g/l |
| $CoCl_2.6H_2O$ | 0.02 | " |
| $H_3BO_3$ | 0.02 | " |
| $(NH_4)_6.Mo_7O_{24}.7H_2O$ | 0.02 | " |

2.   Dissolved ingredients from step 1 in 100 ml. distilled carbon-filtered water.

3.   Autoclaved at 15 lbs for 15 minutes; stored at $13^oC$.

4.   Used 1 ml. stock solution per liter medium.

Stock Vitamin Solution:

1.   
| | | |
|---|---|---|
| D-Biotin | 0.001 | g/l |
| I-Inositol | 1.0 | " |
| Nicotinuric acid | 0.2 | " |
| P-Aminobenzoic acid | 0.02 | " |
| Pantothenol | 0.1 | " |
| Pyridoxine-HCl | 0.02 | " |
| Riboflavin | 0.1 | " |
| Thiamine-HCl | 0.02 | " |

2.   Dissolved ingredients from step 1 in 400 ml. 20% ethyl alcohol.

3.   Stored at room temperature.

4.   Used 2 ml stock solution per liter medium.