Aquaculture, 55 (1986) 249—262
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

A Significant New Systemic Disease of Net-Pen Reared Chinook Salmon (*Oncorhynchus tshawytscha*) Brood Stock

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(Accepted 21 April 1986)

ABSTRACT

Harrell, L.W., Elston, R.A., Scott, T.M. and Wilkinson, M.T., 1986. A significant new systemic disease of net-pen reared chinook salmon (*Oncorhynchus tshawytscha*) brood stock. *Aquaculture*, 55: 249—262.

During an 8-month period in 1983 and 1984, over 80% mortality occurred in groups of 3-year-old chinook salmon (*Oncorhynchus tshawytscha*) brood stock which were being cultured in net-pens in Puget Sound, Washington. Despite thorough pathological and microbiological examination, these losses could not be attributed to known pathogens or poor nutrition. All sick fish were anemic with a marked lymphocytosis. The kidneys and spleens were enlarged at necropsy. Both light- and electron-microscopic examination of spleen and kidney tissue revealed numerous intracellular 3–7-μm spherical organisms with histochemical and ultrastructural characteristics suggesting that they had affinities to marine algae or fungi. The laboratory and field observations indicate that the disease reported here is a previously unknown and significant infectious disease of chinook salmon. Furthermore, this is the first report of a systemic disease of fish caused by an apparently obligate intracellular eukaryotic pathogen. The presumptive causative micro-organism was descriptively termed the chinook salmon rosette agent.

INTRODUCTION

Since 1970, the National Marine Fisheries Service's (NMFS) Manchester Marine Experimental Station has been engaged in rearing captive brood stocks of Atlantic (*Salmo salar*) and Pacific (*Oncorhynchus* spp.) salmon in seawater net-pens (Novotny, 1975). The Snake River stock of chinook salmon (*O. tshawytscha*) is an integral component of NMFS brood-stock programs (Har-

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rell et al., 1985). The efforts of NMFS are directed toward enhancement of depleted natural runs of salmon through development of an egg-bank. Fish are retained in seawater net-pens until maturity; resultant eggs are made available for enhancement and research. Holding salmon in net-pens throughout their marine life affords a unique opportunity to monitor fish health during their adult period, when fish are inaccessible for observation in their wild state.

Approximately 6800 Snake River chinook salmon smolts were first transferred to seawater in April 1982 (as 13-month-old fish). During the following 15 months in seawater, losses to known diseases such as vibriosis (*Vibrio anguillarum*) and bacterial kidney disease (BKD) (*Renibacterium salmoninarum*) were moderated with chemotherapeutics and immunization (Novotny and Harrell, 1977). In August 1983, however, a significant and continuing mortality of 2.5-year-old fish began which could not be attributed to known pathogens, and which eventually resulted in the loss of over 80% of the remaining fish. This paper describes the clinical, histopathological, and ultrastructural characteristics of the disease and its significance to chinook salmon culture.

MATERIALS AND METHODS

Chinook salmon brood-stock husbandry

Adult brood stock were held in 5 m × 5 m × 3-m deep net-pens at a density of 5 kg/m^3. Seawater temperatures ranged from 8 to 12.5°C during the epizootic; mean salinity was 28‰. Fish were fed pelleted rations from several commercial manufacturers supplemented with fresh-frozen herring (*Clupea harengus*) and whole krill (*Euphausia pacifica*). All fish were injected intraperitoneally with a vibrio bacterin/oxytetracycline mixture at 6- to 8-month intervals during their seawater residence. The salmon were also fed chloramphenicol (3 g/kg feed) during epizootics of bacterial disease. Dead and moribund fish were removed from the population daily, weighed and measured, and necropsies performed.

Pathology

Samples of kidney and spleen tissue were aseptically streaked on typicase soy or Sabouraud dextrose agar with 1% fetal bovine serum for determination of the presence of conventional bacterial or fungal pathogens. Cultures were incubated at 15°C. These tissues were also used to prepare wet mounts and smears for staining with Gram stain, or with a modified Wright stain (DIFF-Quik stain set, American Scientific Products, McGaw Park, IL)[1].

Moribund fish were dissected and major organ systems fixed in Bouin's solu-

[1]Reference to trade names does not imply endorsement by the National Fisheries Service, NOAA.

tion and either paraffin-embedded or processed in a plastic histological embedding medium (Elston et al., 1982). Tissue sections were stained with Harris hematoxylin and eosin (H+E), periodic acid Schiff (PAS), Grocott's methenamine silver nitrate (GMS), or Lugol's iodine.

Hematology

Blood was sampled from caudal vessels with heparinized 3-ml glass syringes, and whole-blood smears were prepared on microscope slides and stained with DIFF-Quik. Samples were centrifuged in microhematocrit tubes at 10 000 r.p.m. for 3 min; percent packed cell volumes was recorded. Hemoglobin values were obtained with an American optical hemoglobinometer.

Electron microscopy

Tissues for electron microscopy were fixed in 4% gluteraldehyde in 0.1 *M* sodium cacodylate adjusted to pH 7.4. Tissues were post-fixed with 1.0% OsO_4 in the same buffer for 1 h. Tissues were dehydrated through an ethanol series and embedded in Medcast resin (Pelco, Tustin, CA). The thin sections were routinely stained with uranyl acetate and lead citrate and examined with a Phillips EM 300 at an accelerating voltage of 60 kV. Both the histological and electron microscopical examinations included systematic searches of affected tissues (e.g., spleen and kidney) for the presence of viral replication or other infectious and noninfectious processes.

RESULTS

Approximately 16 months after seawater entry (at approximately 2.5 years of age), mortality in the Snake River chinook salmon began to exceed 0.5% per day (Fig. 1). Necropsy of dead and moribund fish revealed swollen kidneys and spleens (Fig. 2). Although considerable losses among males were attributed to early maturation (Fig. 1), these fish, as well as larger numbers of normally maturing fish, exhibited uniform signs of a severe pathological condition. Sick fish were severely anemic, with an average packed cell volume of 12% and hemoglobin values of 4.9 ml/dl. Whole-blood smears stained with DIFF-Quik indicated a pronounced lymphocytosis. Kidney and spleen tissue streaked on typicase soy and Sabouraud dextrose agar plates were negative for bacterial or fungal growth. Gram-stained smears of these tissues were negative for *R. salmoninarum*, the causative bacterium of BKD. Exhaustive examination of spleen, kidney, and blood by electron microscopy did not reveal the presence of viral particles. However, clusters of Gram-positive staining, spherical (3–7 μm in diameter) organisms were observed (Fig. 3a). Tissue smears stained with DIFF-Quik revealed that the clusters of organisms had a characteristic periph-

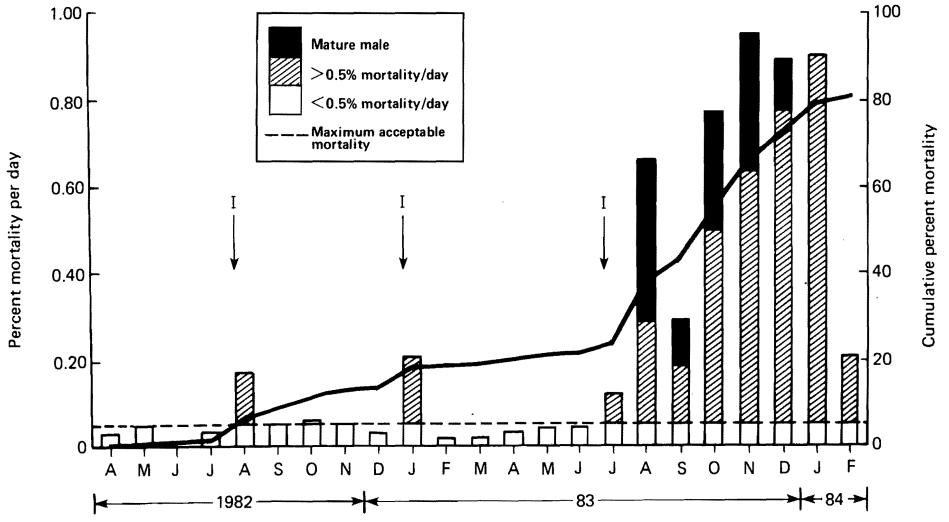

Fig. 1. Mortality of 1980-brood Snake River fall chinook salmon in marine net-pens. Losses at I indicate inventory discrepancies and mortality from handling. The solid dark bars indicate mortalities which were attributed to early maturation of male fish, although these fish were also infected with the rosette organisms. The horizontal axis indicates the month and year of each sample.

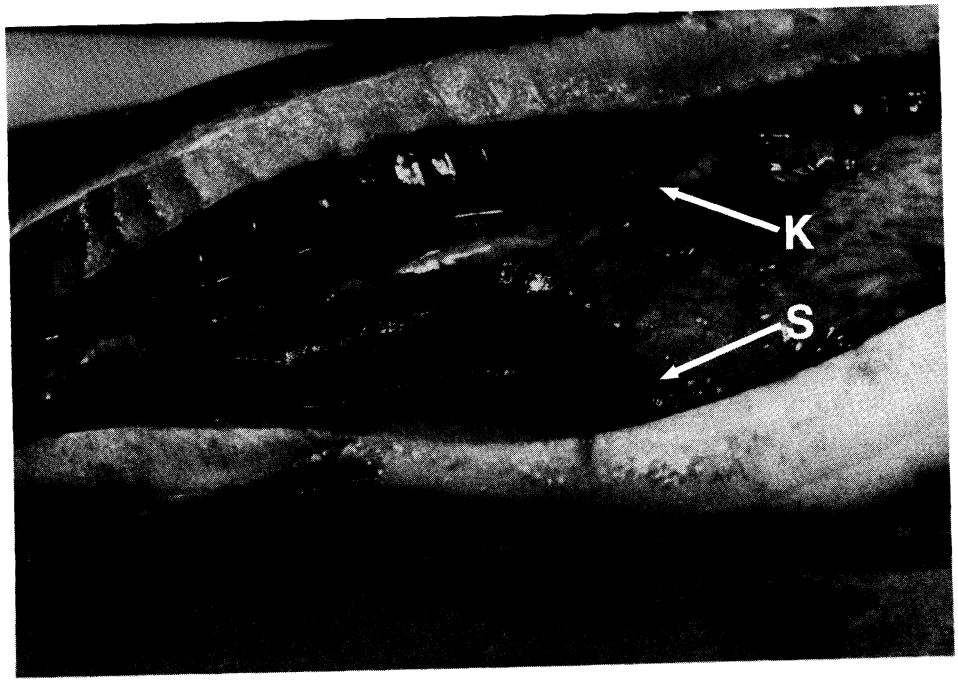

Fig. 2. Enlarged kidney (K) and spleen (S) in rosette-infected chinook salmon.

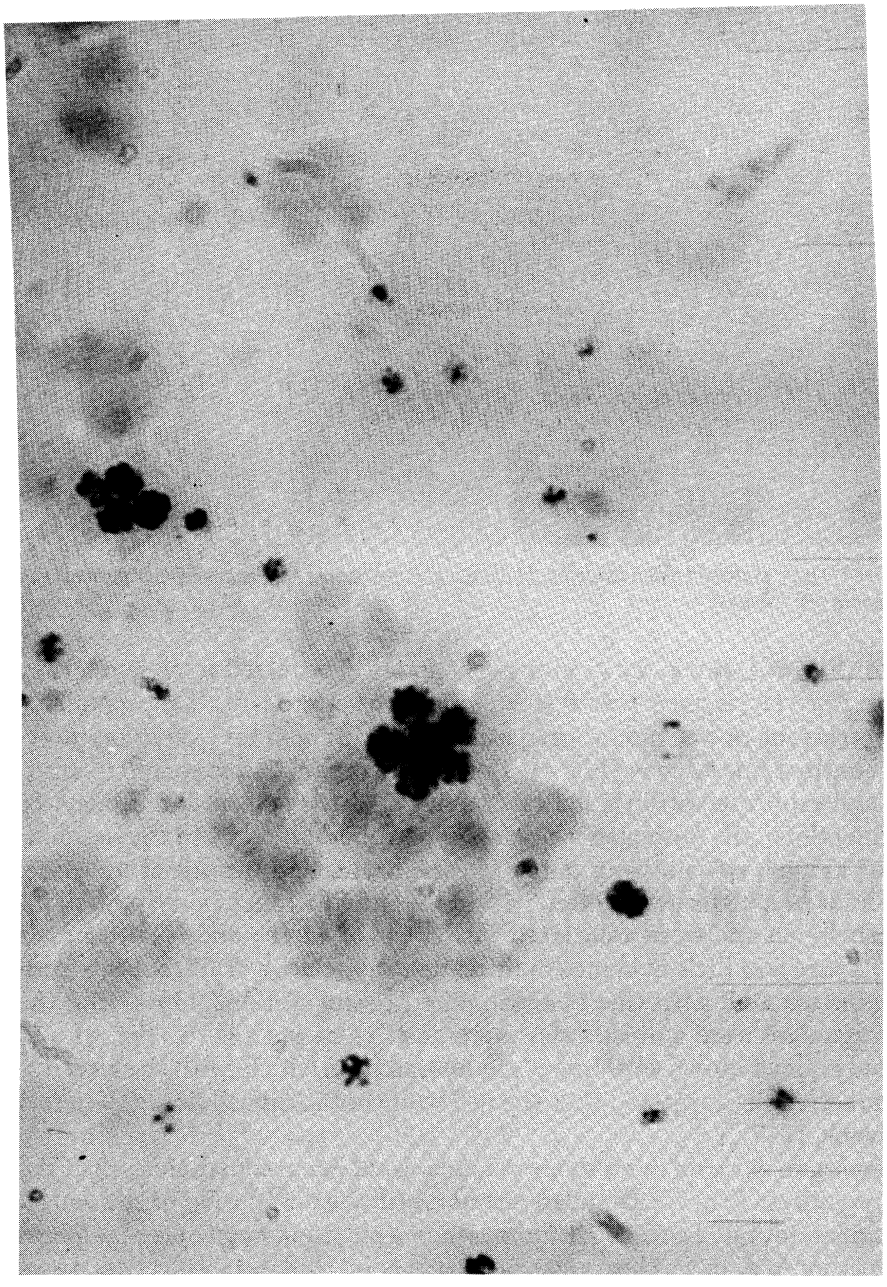

Fig. 3a. Gram-stained imprint of spleen from infected chinook salmon. Note the cluster of six organisms near the center of photomicrograph (×400).

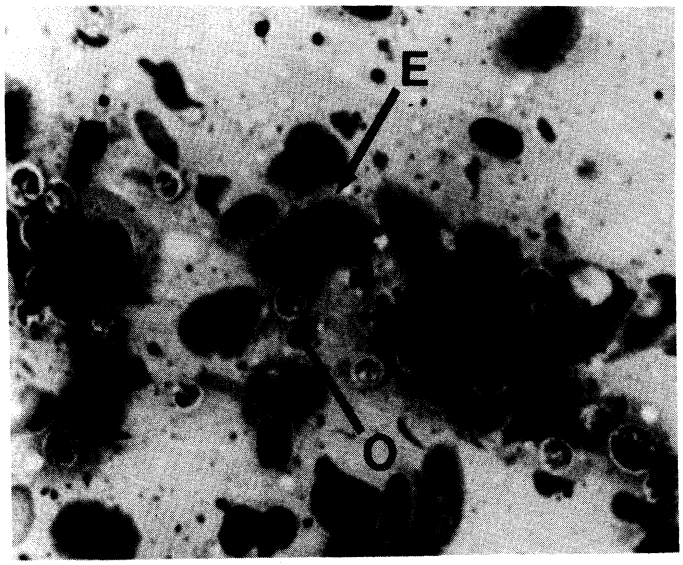

Fig. 3b. DIFF-Quik-stained spleen imprint. Note the size relationship of causative organism (O) to erythrocyte (E) (×400).

eral halo (Fig. 3b). Since the organisms constantly occurred in clusters, they were descriptively termed rosettes or chinook rosette agent. The organisms were birefringent in wet-mount preparations as observed by Nomarksi interference contrast microscopy (Fig. 4). The rosette organisms appeared to accumulate and replicate within fixed macrophages of the spleen and kidney. In severe infections, the pathogen was seen in peripheral blood and the vascular spaces of kidney, spleen, liver, gonad, heart, brain, and intestinal mucosae. Intracellular organisms were found within the interstitium of spleen and kidney parenchyma and were associated with areas of edema and focal necrosis (Fig. 5). There was little evidence of inflammatory change or fibroblastic proliferation associated with this disease. When tissue sections containing the rosette organism were stained, they were positive to both PAS (Fig. 6) and GMS, and stained brown with Lugol's iodine solution which, with the birefringent characteristic, suggested that the cell wall contained cellulose (Bancroft and Stevens, 1977).

Transmission electron microscopy demonstrated intracellular clusters of the rosette organisms (Fig. 7). Detailed ultrastructural examination of the causative organism revealed a cell wall composed of a single-layered outer membranous structure (possibly of host cell origin); a second moderately electron dense layer; and a thin, inner electronlucent zone that separated the second layer from the plasma membrane (Fig. 8). The organisms contained peripherally-oriented mitochondria within a ribosomal matrix and contained both membrane-bound and nonmembrane-bound vacuoles of varying density (Fig. 9).

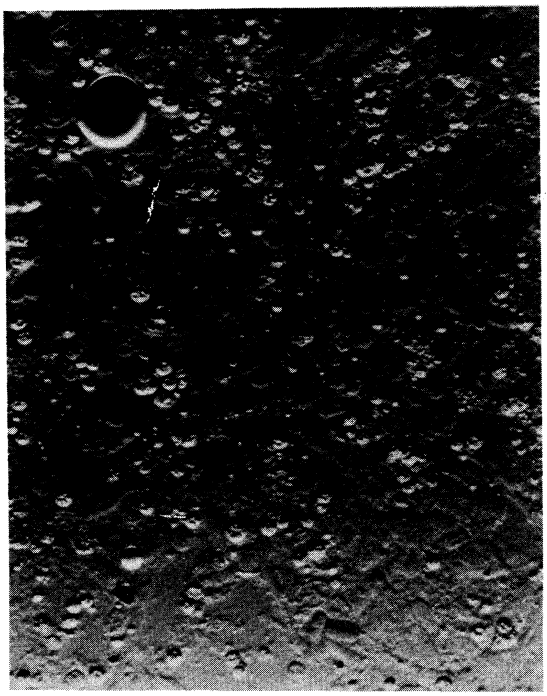

Fig. 4. Rosette organisms from spleen of infected chinook salmon. Nomarksi interference contrast (×400).

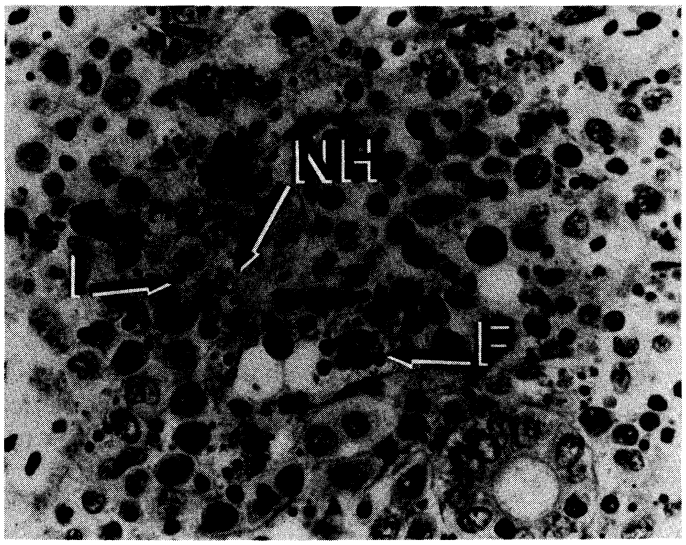

Fig. 5. Necrotic hematopoetic tissue (NH) associated with free (F) and intracellular (I) organisms within posterior kidney of infected chinook salmon (H+E, ×400).

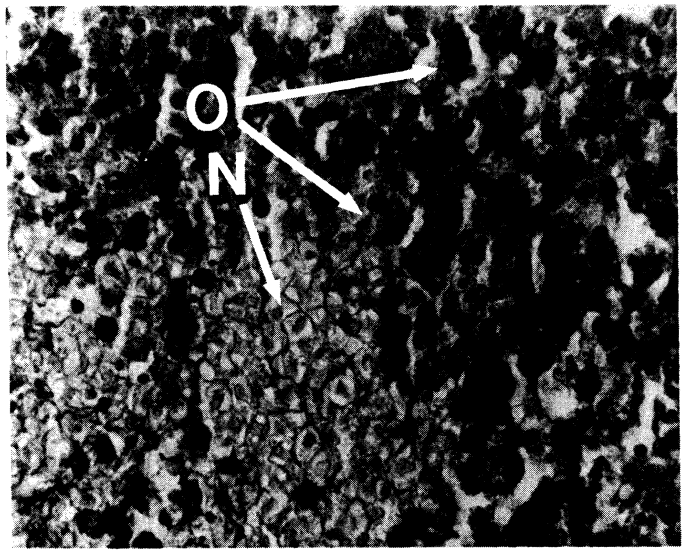

Fig. 6. Section of spleen with proliferation of rosette organisms (O), normal parenchyma (N) (PAS, ×1000).

Nuclei were relatively indistinct. Partition of the organisms (Fig. 10) as a means of apparent vegetative reproduction was observed.

DISCUSSION

The results of this study provide substantial and diverse presumptive evidence to link the infectious rosette organism and the salmon mortalities. The intracellular pathogen's invasive nature and the associated anemia could clearly lead to the observed morbidities and mortalities. Except for the limited macrophage activity and edema, there was negligible tissue reaction in the form of inflammation or granulation. This minimal host response could be attributed to an immunologically inert cellulose cell wall of the pathogen. The lymphocytosis appeared to be a limited inflammatory response to the pathogen. The infection, however, appeared to overwhelm chinook salmon before any effective defense mechanism was established.

Three broad categories of identity of the rosette organism could be considered: algal, fungal, or protozoan. Protozoa include seven phyla (Levine, 1980) to which the organism was initially compared. Ultrastructural characteristics appear to eliminate a relationship of the disease organism to most of the protozoan phyla. For example, the organism described here contained mitochondria, and these organelles are absent in the microspora (Sprague, 1982). Other specific ultrastructural characteristics (e.g., apical complex or polar filaments) which would link the new organism to other protozoan phyla such as the Apicomplexa, Myxozoa, or Ascetospora were not observed, although it is possible

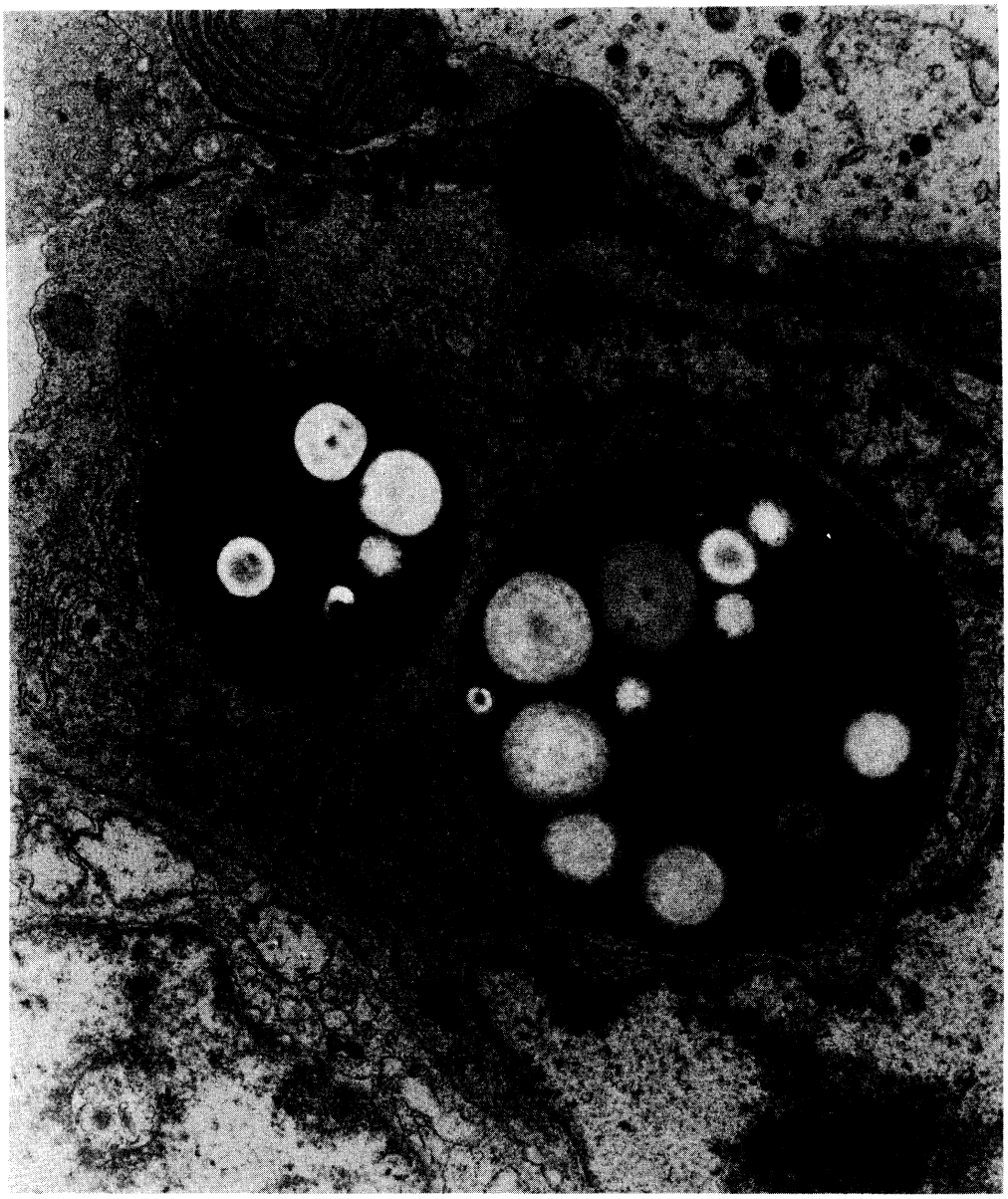

Fig. 7. Intracellular organisms in kidney tissue; note nucleus (N) of chinook salmon kidney cell (×17 650).

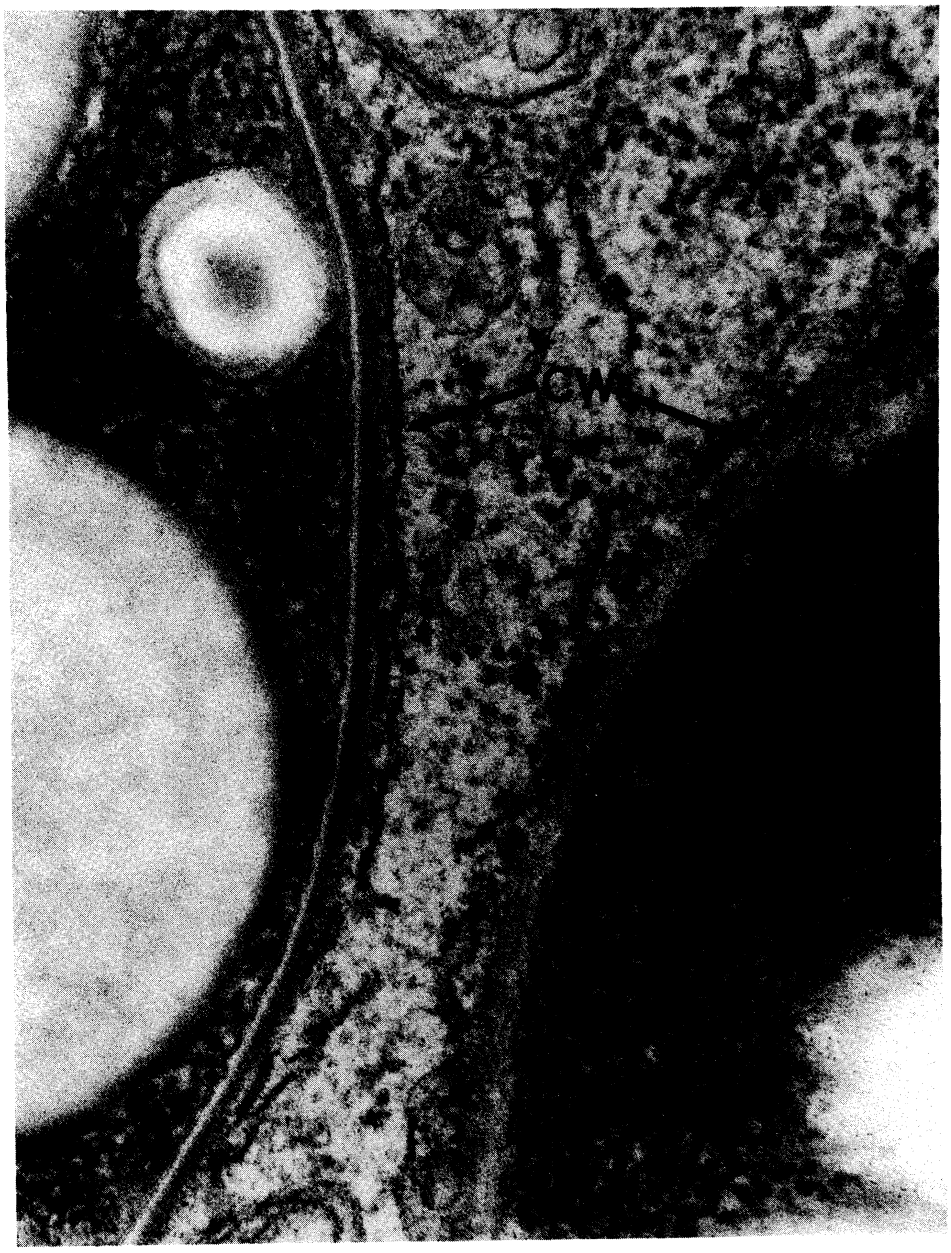

Fig. 8. Multilayered cell wall (CW) of rosette organism (×156 000).

that an as yet unobserved life-cycle stage could contain such definitive structures. Organisms considered to have fungal affinities are known to have cell walls composed of chitin or cellulose or a combination thereof (Barr, 1982). Taxonomic affinities of the microscopic forms of true fungi (Eumycota), such

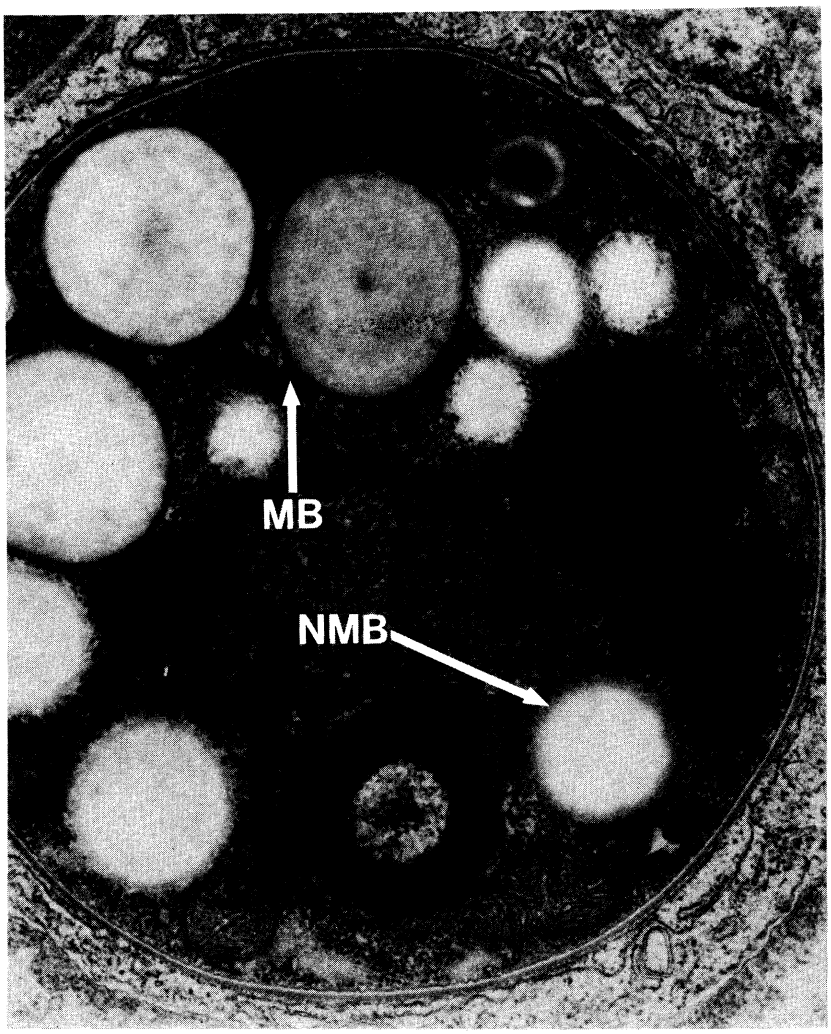

Fig. 9. Membrane-bound (MB) and nonmembrane-bound (NMB) vacuoles of varying density within causative organism (×30 850).

as the Chitridiomycetes, are largely determined by characteristics of the zoosporic stages or of the reproductive structure. Some organisms, such as *Aplanochytrium* spp., however, apparently do not exhibit a zoosporic stage. Other taxonomically problematic organisms, such as *Hyalochlorella marina*, divide only by continuous internal divisions of the vegetative cells (Alderman, 1974). This latter organism is considered to be a colorless derivative of the chlorophyte *Chlorella* spp. (Poyton, 1970). In view of these considerations, the salmon parasite described in this paper may be a previously undescribed parasitic

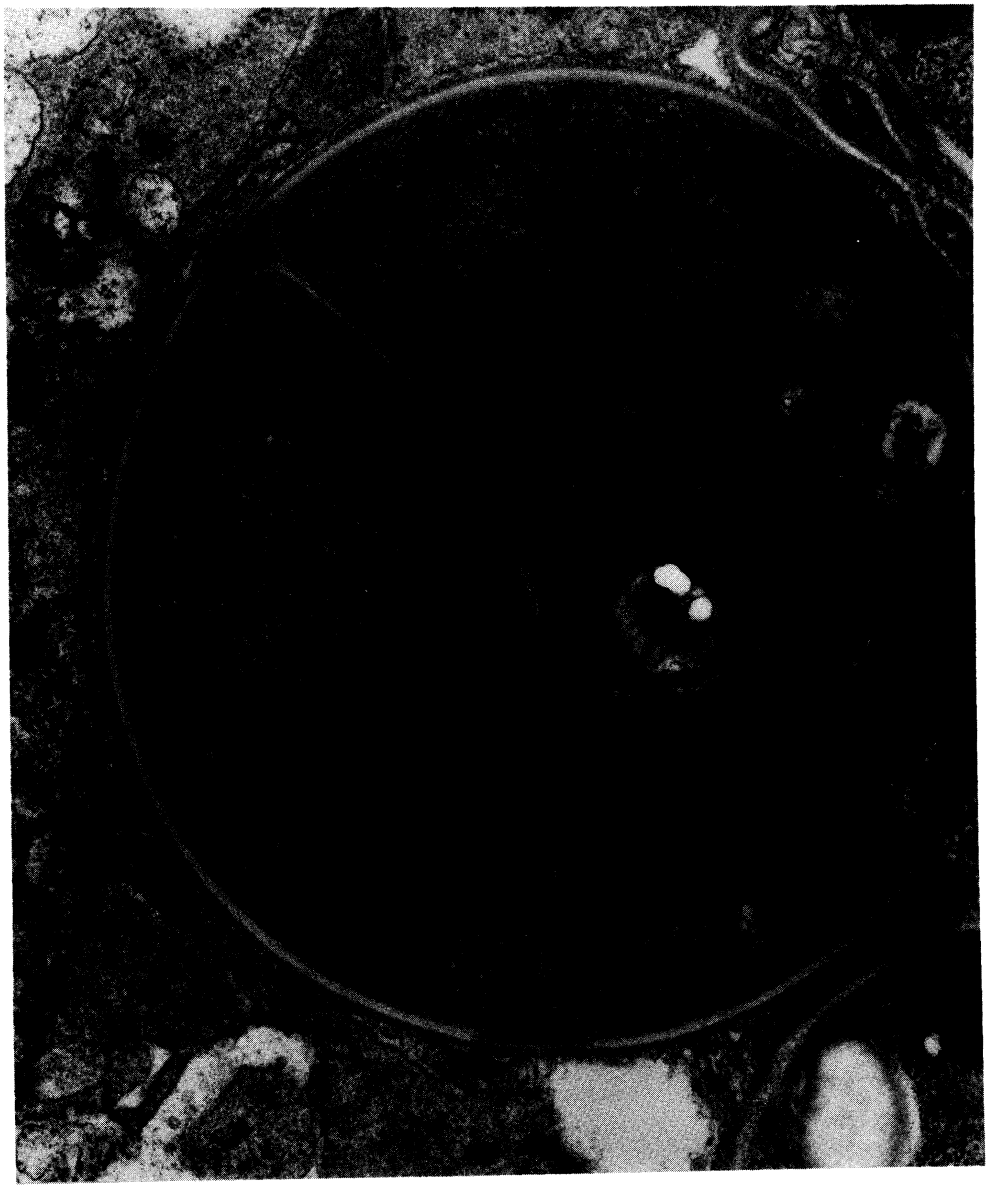

Fig. 10. Partition of the rosette organism (×36 850).

derivative of simple marine algae or fungi. Further studies will be required to determine if a zoosporic stage exists. At present, the taxonomic affinity is problematic, as with similar simple marine coccoid forms, and will remain so until further biochemical, structural, and developmental information is available and a higher taxonomic structure for such focus is established.

Other investigations have described chronic mycotic diseases of salmonids.

Miyazaki and Kubota (1977) described a chronic inflammatory response of rainbow trout (*Salmo gairdneri*) infected with the marine fungus *Ichthyophonus hoferi*. Similarly, *Exophilia salmonis* infections in marine-reared Atlantic salmon induced a chronic inflammatory response and extensive granulomata (Richards et al., 1978). Both of the afore-mentioned fungal diseases were associated with the feeding of raw marine fish. Our chinook salmon were fed supplements of both raw herring and krill; however, no evidence of the chinook salmon pathogen was observed by microscopic examinations of either supplement.

It is improbable that the rosette agent is a freshwater pathogen. Routine Gram staining of kidney and spleen tissues is a common practice of Pacific Northwest hatchery biologists, and it would be difficult to fail to recognize this new organism. Also, the authors have microscopically examined over 200 presmolt Snake River chinook salmon and have seen no evidence of infection.

Further studies on the potential occurrence of the rosette organism in wild salmon, other fish species or marine invertebrate species utilized as supplemental foodstuffs will be the subject of future experimentation with chinook salmon at the NMFS Manchester Experimental Station. The obligate intracellular nature of the rosette organism and resultant mortality strongly suggest that this disease is a significant threat to the marine rearing of chinook salmon brood stock and possibly to wild and hatchery-produced stocks of anadromous fish.

ACKNOWLEDGEMENTS

This study was supported in part by the Bonneville Power Administration (BPA), Portland, OR. Richard Harper, BPA contract officer, provided valuable and constant assistance. We also thank Glenn Hoffman, Parasitologist, U.S. Fish and Wildlife Service, Stuttgart, AR, for his timely guidance and encouragement.

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