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Systemic hexamitid (Protozoa: Diplomonadida) infection in seawater pen-reared chinook salmon *Oncorhynchus tshawytscha*

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ABSTRACT: A systemic infection with a hexamitid flagellate resembling *Hexamita salmonis* caused high mortality in chinook salmon *Oncorhynchus tshawytscha* reared at a seawater netpen farm in British Columbia, Canada. Affected fish were anemic and had swollen abdomens containing serosanguinous ascites and large blood clots. They also had an enlarged, mottled and congested liver, and an enlarged kidney and spleen. Numerous parasites were observed in the blood. The most remarkable histological changes were found in the liver and kidney. Livers of affected fish showed edema, congestion and inflammation. The renal interstitium was moderately hyperplastic due to proliferation of hemoblasts. The systemic infection was transmitted in the laboratory to chinook by intraperitoneal injection, by gavage of infected ascites and by waterborne exposure (in both fresh and sea water) with a mixture of infected ascites and tissue. The infection was also transmitted in fresh and sea water by cohabitation with infected chinook. Atlantic salmon were refractory to the infection. Based on the ease of transmission of the parasite in both fresh and sea water, and the high mortality associated with the infection, this disease poses a potentially serious threat to aquaculture of chinook salmon.

INTRODUCTION

A systemic infection of a hexamitid flagellate (family Hexamitidae) caused high mortality in chinook salmon *Oncorhynchus tshawytscha* reared in seawater netpens in British Columbia, Canada. The parasite closely resembled *Hexamita salmonis*, a common parasite of the intestinal tract of freshwater-reared salmonids (Becker 1977). Some reports have attributed anorexia, emaciation, poor growth and mortality in salmon fry to the gut infection (Davis 1926, Sano 1970, Becker 1977), whereas others have suggested that the parasite is, at best, a questionable pathogen (Uzman et al. 1965). Based on numerous diagnostic cases from freshwater hatcheries, gut infections by *H. salmonis* are considered to be essentially non-pathogenic in British Columbia (G. E. Hoskins, Pacific Biological Station, Nanaimo, B. C., Canada, pers. comm.).

Other hexamitid parasites (*Hexamita and Spironucleus* spp.) infect strictly marine fishes (Lavier 1936, Kulda & Lom 1964, Poynton & Morrison 1990, Woo & Poynton 1992), and Lom (1984) suggested that *Hexamita salmonis* may persist in salmonids after they migrate to sea water. There are few reports of systemic and other extra-intestinal infections by hexamitid parasites in fishes. Molnár (1974) reported *Spironucleus* sp. in several species of freshwater fishes. The parasites were observed in blood vessels and interstitial hemorrhages in the skeletal muscle of unspecified aquarium fishes, and liver infections were detected in *Barbus barbus*. Ferguson & Moccia (1980) reported a disseminated infection by a hexamitid flagellate in Siamese fighting fish *Betta splendens*, and the parasite was found in peritoneal exudate and the parenchyma of the liver, spleen and kidney. Mo et al. (1990) and Poppe (1992) described systemic hexamitosis in netpen-reared Atlantic salmon *Salmo salar* from Norway which caused granulomatous lesions. We describe here the

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clinical, gross, and histopathogical changes associated with a severe systemic infection of a *H. salmonis*-like parasite in chinook salmon. We also describe the morphology of the parasite and experimental transmission of the disease.

MATERIALS AND METHODS

Clinical observations. Unusual mortality was noted in chinook salmon at a netpen farm in the Sechelt area, British Columbia, in September 1991. These fish had been introduced to sea water in spring 1990, and henceforth are referred to as the 1990 lot. The fish weighed ca 1 to 2 kg at the time of first examination in October 1991. Four moribund fish were examined on 18 October 1991, and 10 moribund fish on 21 October 1991. To determine the prevalence of the infection in the 1990 lot, the livers and intestine of 20 apparently healthy fish were examined using wet mount preparations on 24 October, 1 and 25 November 1991. The prevalence of the infection in chinook introduced to this netpen farm in summer 1991 (the 1991 lot) was assessed by examining samples of 20 apparently healthy fish on 24 October 1991 and 15 February 1992.

Examination for concurrent bacterial infections was accomplished by aseptically obtaining inocula from the kidneys of 6 severely affected fish from the 1990 lot and plating on Tryptic Soy Agar supplemented with 1% NaCl. Examination for concurrent viral infections in moribund fish from the 1990 lot was done by inoculating CHSE-214 and EPC cell lines with ascites from 5 fish using standard techniques (Amos 1985). The inoculated tissue cultures were maintained at 10 and 15 °C for 2 wk. In addition, infected livers were examined by transmission electron microscopy for the presence of virus particles (see below).

Microscopy. Wet mount preparations were made of the intestinal mucosa, liver, ascites, blood and skin from moribund fish. The parasite in wet mounts was measured and photographed immediately after immobilization with dilute formalin, the formalin being added to one edge of the wet mount preparation. As suggested by Davis (1926), fresh and 3 d old intestinal contents were examined in wet mounts stained with Lugol's iodine for the presence of cysts. Blood and intestinal smears, and liver imprints were stained with Leishman's Giemsa (Yasutake & Wales 1983). The gills, brain and visceral organs were fixed in Davidson's solution (Humason 1979), processed for histological examination and stained with hematoxylin and eosin. For transmission electron microscopy (TEM), liver tissue was fixed in 4% glutaraldehyde in Millonig's buffer and processed using standard techniques. Liver and blood specimens for scanning electron microscopy (SEM) were fixed in 4% glutaraldehyde in Millonig's buffer, critical point dried in CO_2 and sputter coated with AuPd.

Experimental transmission. Transmissibility of the flagellate to chinook salmon (average wt 150 g) was assessed by intraperitoneal (IP) injection, by gavage with infected ascites, by waterborne exposure to infected tissue and ascites (henceforth referred to as waterborne exposure) and by cohabitation with infected fish. These experiments were conducted in 200 l open system seawater tanks at ca 10 °C. Transmission by cohabitation and waterborne exposure was also assessed in 200 l open system freshwater tanks at ca 8 °C. In addition, the susceptibility of Atlantic salmon *Salmo salar* (average wt 75 g) to the infection was assessed by gavage and the fish were held in sea water as described above. All recipient salmon used for these experiments were obtained from stocks held at the Pacific Biological Station, Nanaimo, British Columbia, a site where the systemic infection had never been detected.

IP injection: Infected ascites fluid was obtained from the 1990 lot of chinook for IP injection. Fifteen chinook were injected with 0.3 ml of ascites, which contained 6.2×10^6 flagellates ml^{-1}.

Gavage: Infectivity of the parasite for chinook and Atlantic salmon by ingestion was assessed by gavaging 15 fish of each species with 1 ml of infected ascites containing ca 2×10^6 flagellates ml^{-1}. Infected ascites fluid used in this experiment was obtained from a chinook infected by cohabitation exposure.

Waterborne exposure: Waterborne exposure was performed by adding 300 ml of heavily infected ascites and chopped liver, gut and kidney to freshwater and seawater tanks (each containing 15 uninfected chinook) at the beginning of the experiment and 2 d later. During each exposure, the water flow was turned off for 1 h and then turned on to ca 5 l min^{-1}.

Cohabitation: Exposure by cohabitation was accomplished by placing 15 uninfected chinook with 5 infected chinook in a freshwater tank, and with 9 infected fish in a seawater tank. The infected fish had been exposed by gavage of infected ascites as described above.

***In vitro* parasite survival.** The survival of the parasite in water of different salinities was investigated by adding 0.5 ml of heavily infected ascites to 9.5 ml sea water, fresh water (well water) or 0.85% NaCl, each maintained at 10 °C. Viability of the parasites in these environments over time was assessed by observing the organisms for motility and movement of flagella in wet mount preparations examined with phase contrast microscopy at 5 and 30 min, 1, 2, 3 and 4 h, and 1, 2 and 3 d. As a control, infected ascites (maintained at 10 °C) was examined daily for 3 d.

RESULTS

Clinical observations

Unusually high mortality in the 1990 lot of chinook was first noted by fish farmers in September 1991, and continued through autumn 1991 and winter 1992. During October, the 1990 lot exhibited 18% cumulative mortality, the most severely affected pen having 43% mortality. Essentially all moribund fish from this population examined by us and by the fish farmers during this month revealed massive, systemic infections of the flagellate. Therefore, virtually all of the mortality was attributed to infection with the parasite. After October 1991, blooms of the diatom *Chaetoceros convolutus* and frequent attacks by harbor seals *Phoca vitulina* also caused high mortality in the 1990 lot. Thus, although the flagellate infection was still prevalent in the population, it was not possible to determine its contribution to the overall mortality on the farm after October 1991

Fish with the systemic flagellate infection were lethargic and accumulated near the surface. Affected fish were dark in color and some had a distended, swollen abdomen. The gills were pale due to anemia. Internal examination of affected fish consistently revealed extremely hypertrophied livers, serosanguinous ascites, blood clots and chicken fat clots (clots largely devoid of erythrocytes). The liver was congested and mottled, and usually exhibited numerous petechiae and large, multifocal, whitish, friable lesions. The spleen and kidney were moderately enlarged, and petechiae occurred throughout the skeletal muscle. Infections by pathogenic bacteria or viruses were not detected in the affected fish.

Examination of 60 apparently healthy fish from the 1990 lot in late October and November 1991 revealed a 48% prevalence of the infection. The parasite was detected in the livers and lower intestine of 21 fish, 7 fish had intestinal infections only and no parasites were detected in the remaining 32 fish. In the 1991 lot, only 1 of the 20 apparently healthy chinook examined in October 1991 revealed the infection, and the parasite was confined to the intestine. The parasite was not detected in apparently healthy fish from the 1991 lot examined in February 1992. However, the infection was detected in several dead or moribund fish from the 1991 lot in October 1991 and February 1992.

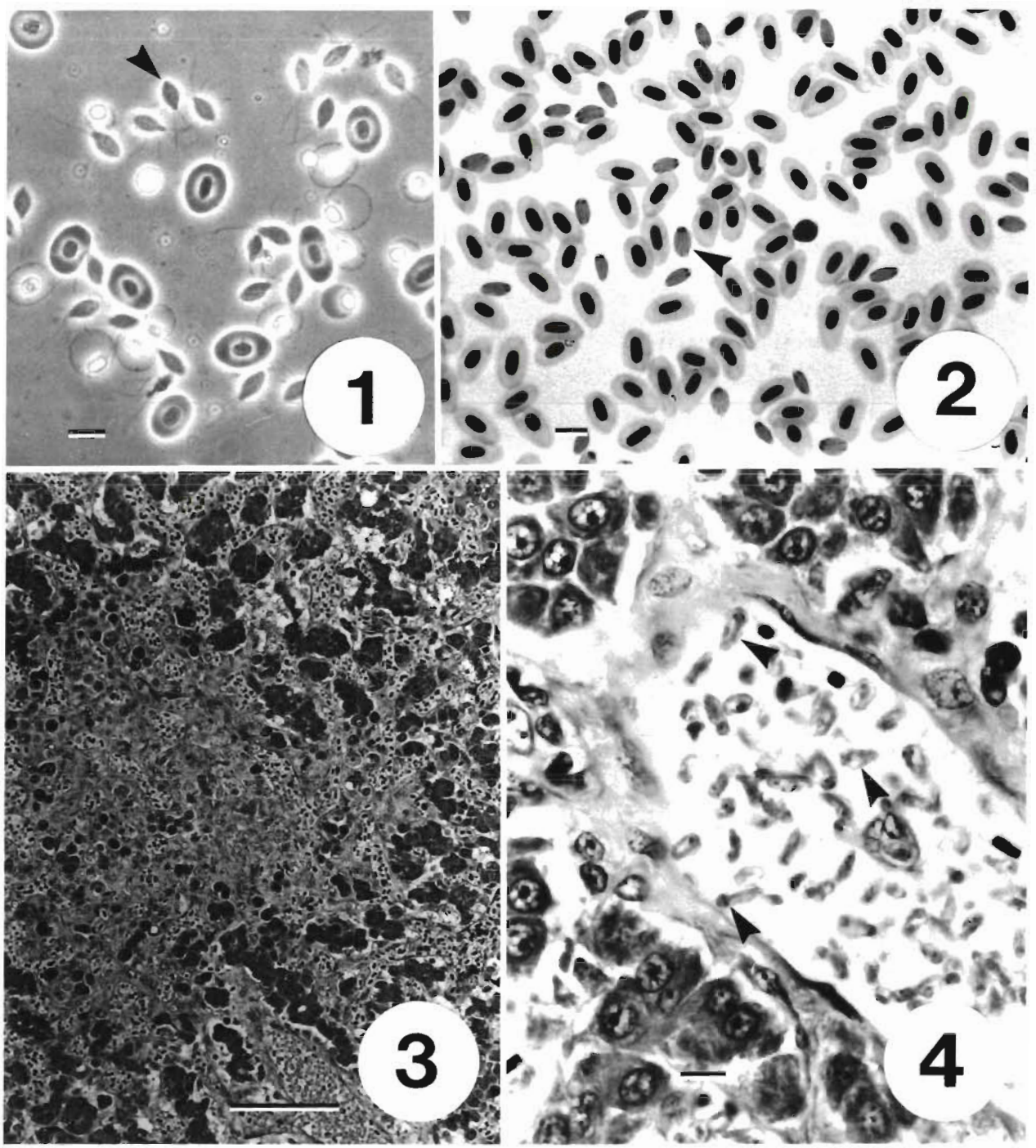
Microscopy

Wet mount preparations of the liver, kidney and lower intestine contained massive numbers of a highly motile, broadly pyriform flagellate that tapered at the posterior end (Fig. 1). The parasite measured 10.5×5.5 µm (n = 20). Moderate numbers of the organism were observed in the mucus from skin scrapings. The organism had 3 pairs of flagella that emerged anterior-medially and one pair of recurrent flagella that emerged at the posterior end. The organism was readily detected in liver and kidney imprints and in blood and intestinal smears stained with Leishman's Giemsa (Fig. 2). In these preparations, the flagellate was oval, dark-staining, and had 2 separate, clear, funnel-shaped bands (representing the flagellar pockets), which extended the length of the organism. Two red-staining nuclei at the anterior end of the organism were visible in most preparations. The ovoid nuclei contained a prominent karyosome, separated from the nuclear membrane by a clear zone.

Histological examination revealed massive numbers of the flagellate in the blood vessels of essentially all organs, including the liver, kidney, spleen, intestine, pancreas, heart, brain and gills (Figs. 3 to 7). In tissue sections stained with hematoxylin and eosin, the parasite had a clear-staining cytoplasm, and the nuclei and cell membranes were basophilic (Fig. 4). The liver was edematous and congested, and had a diffuse, mild inflammation consisting primarily of cells resembling lymphoblasts and plasmablasts. Large focal areas of necrosis were observed in the liver in some fish (Fig. 3). Major blood vessels and sinusoids of the liver contained numerous parasites. Blood vessels in the pancreas and associated fat were dilated and contained high numbers of the flagellate.

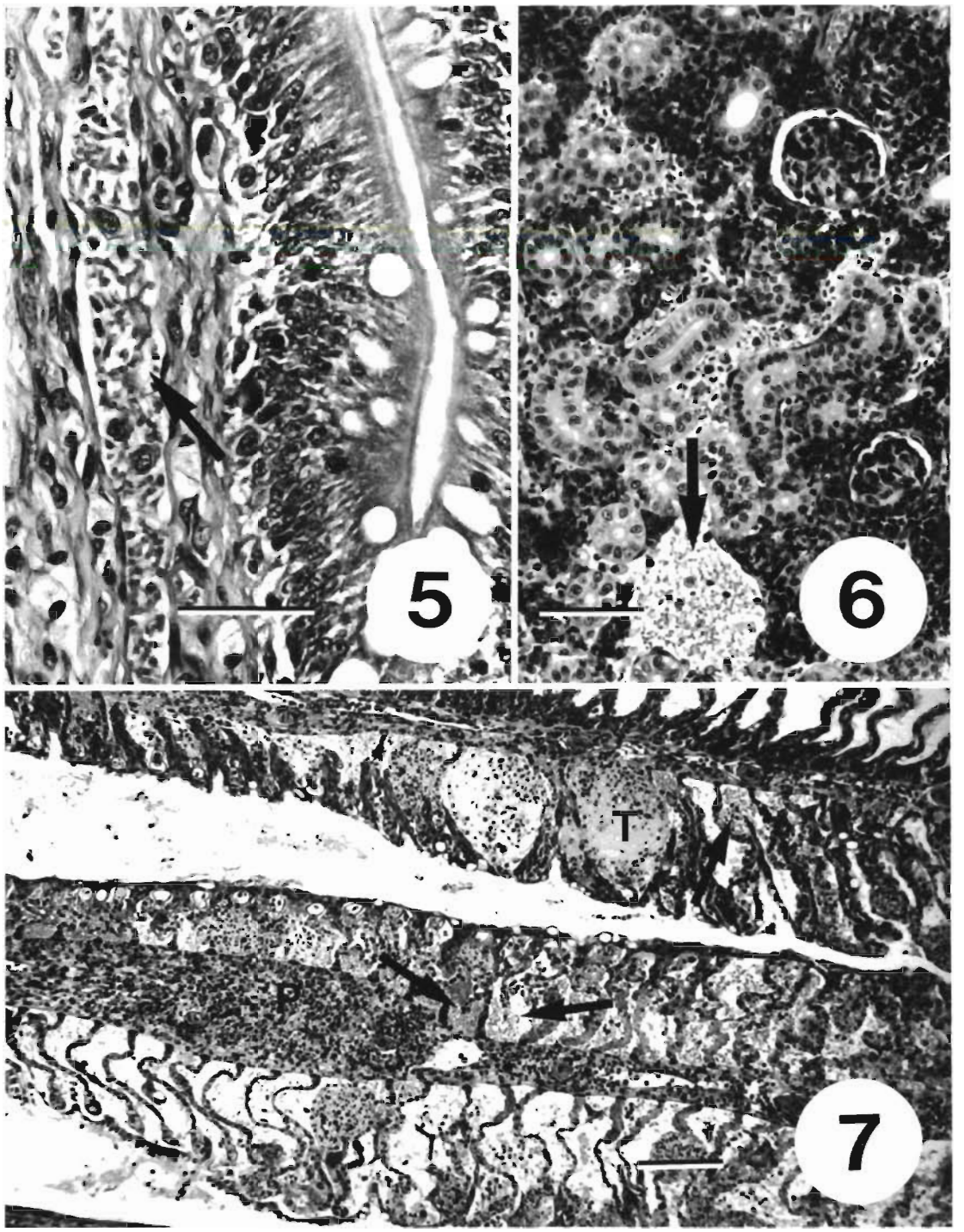
The renal interstitium was moderately hyperplastic due to proliferation of hemoblasts. Large numbers of the flagellate were consistently observed in the large blood vessels in the kidney (Fig. 6). Mild, diffuse necrosis of the hematopoietic tissue was observed in some fish. Kidneys also exhibited mild, generalized proliferative glomerulonephritis, but the renal tubules appeared unaffected. The spleen was extremely congested and large numbers of the parasites occurred in the blood.

The lamina propria of the pyloric caeca and lower intestine exhibited mild, diffuse inflammation, and the blood vessels were dilated and packed with the parasite (Fig. 5). However, the epithelium was intact and exhibited no significant necrotic changes. The flagellate was detected in luminal contents of the gut but was not observed associated with the gut epithelium. The brain appeared normal, but the meninges were edematous and the parasite was detected in the capillaries. The heart was essentially normal, except the pericardium was edematous and the atrial blood was heavily infected. Blood vessels of the gills were also heavily infected, but the gills of most fish were otherwise normal. However, in 3 of the 14 fish from the 1990



Figs. 1 & 2. *Oncorhynchus tshawytscha*. The hexamitid flagellate (arrowheads) in the blood of chinook salmon. Scale bar = 10 μm. Fig. 1. Wet mount preparation, phase contrast. Fig. 2. Leishman's Giemsa

Figs. 3 & 4. *Oncorhynchus tshawytscha*. Liver with hexamitid parasite infection. H & E. Fig. 3. Focal necrosis and heavy infection in liver Scale bar = 50 μm. Fig. 4. The flagellates in blood vessel (arrowheads). Scale bar = 10 μm



Figs. 5 to 7 *Oncorhynchus tshawytscha*. Intestine, kidney, gill with the hexamitid flagellate in blood vessels (arrows). H & E. Fig. 5. Infection of the flagellate in blood vessel of lamina propria in the intestine. Scale bar = 50 μm. Fig. 6. Renal interstitial hyperplasia and proliferative glomerulonephritis. Scale bar = 50 μm. Fig. 7. Gills with edema, telangiectasia (T), and inflammation (P) in the primary lamella. Scale bar = 100 μm

lot, the secondary lamellae were edematous and the blood vessels were dilated (Fig. 7). Gills of these fish also exhibited telangiectasia, and a mixed inflammatory infiltrate comprised of granulocytes, macrophages and lymphocytes in the primary lamellae.

TEM of the flagellate showed that the nuclei were closely apposed in the middle of the cell. Kinetosomal complexes were close to each other, posterior to the nuclei and medial in the cytoplasm (Fig. 8). As observed by light microscopy and SEM, the anterior flagella emerged at the medial, anterior-most aspect of the organism. The recurrent flagella were encased in a flagellar pocket that extended the length of the organism. SEM of the flagellate revealed the same arrangement of the flagella as seen by light microscopy (Fig. 9). Two flagella emerged from a funnel-like opening in the flagellar pocket near the apex of the posterior end, and the opening had no ring-shaped protrusions (tori).

Transmission

The systemic flagellate infection was transmitted to chinook salmon by IP injection and gavage in sea water, and by water-borne exposure and cohabitation in both sea water and fresh water. Infected recipient fish in all experiments exhibited heavy infections of the blood, and identical gross and histological changes as those seen in fish from the netpen site. Examination of 20 unexposed fish from the recipient fish stock revealed no hexamitid infections in either the blood or intestine.

IP injection of chinook. All fish in this group developed heavy systemic infections of the parasite and the disease. The first mortality in the injected chinook occurred 7 d post exposure (PE). At 11 d PE, 10 of the 15 injected fish had died with the disease, and the study was terminated.

Gavage of chinook and Atlantic salmon. All chinook that were exposed by gavage developed the disease. By 14 d PE some of the chinook in the tank were dark and lethargic and the first fish in this group died at 21 d PE. All of the remaining 14 chinook died with the systemic infection by 32 d PE. The Atlantic salmon showed no signs of the disease, and wet mount and histological examination of the blood, liver and intestine of these fish at 32 d PE did not reveal any signs of the infection.

Waterborne exposure of chinook. Fish exposed by this method in fresh water first began to die with the disease 10 d PE. (In this case, PE refers to the time following the initial exposure.) After 24 d, 7 of the 15 fish had died with the systemic infection, and the study was terminated. The remaining fish that were sacrificed at this time also had the infection.

Chinook in the seawater waterborne exposure experiment also became infected. At 14 d PE the fish appeared dark and lethargic. Six of these fish were examined, and all had the systemic infection and internal changes consistent with the disease. At 17 d PE, the first fish in this group died. By 24 d PE an additional 6 fish had died and the experiment was terminated. All of the exposed fish had the disease.

Cohabitation with chinook. All uninfected chinook maintained in fresh water and sea water with infected chinook developed systemic infections. In fresh water, the infection was first detected in a previously uninfected fish at 20 d PE. (In this case PE indicates post-initial exposure.) At 24 d PE, the previously uninfected fish in the seawater tank began to die with the disease, and the experiment was terminated.

In vitro parasite survival

The flagellate survived poorly in fresh water, and after 5 min all of the organisms were motionless, swollen and apparently dead. After 1 h in sea water, all of the flagellates appeared viable, as indicated by movement of flagella, but about 90 % of the organisms were shrunken and non-motile. By 4 h, the flagellates in sea water showed no movement, indicating that they were dead. The flagellate survived better in 0.85 % NaCl, its shape remaining unaltered. After 1 d, about 50 % of the organisms were immotile, although they still showed flagellar movement, while the rest of the organisms were apparently dead. Almost all of the flagellates in this solution were dead after 3 d. The parasite survived best in ascites fluid, and most were actively motile and not distorted in shape after 3 d.

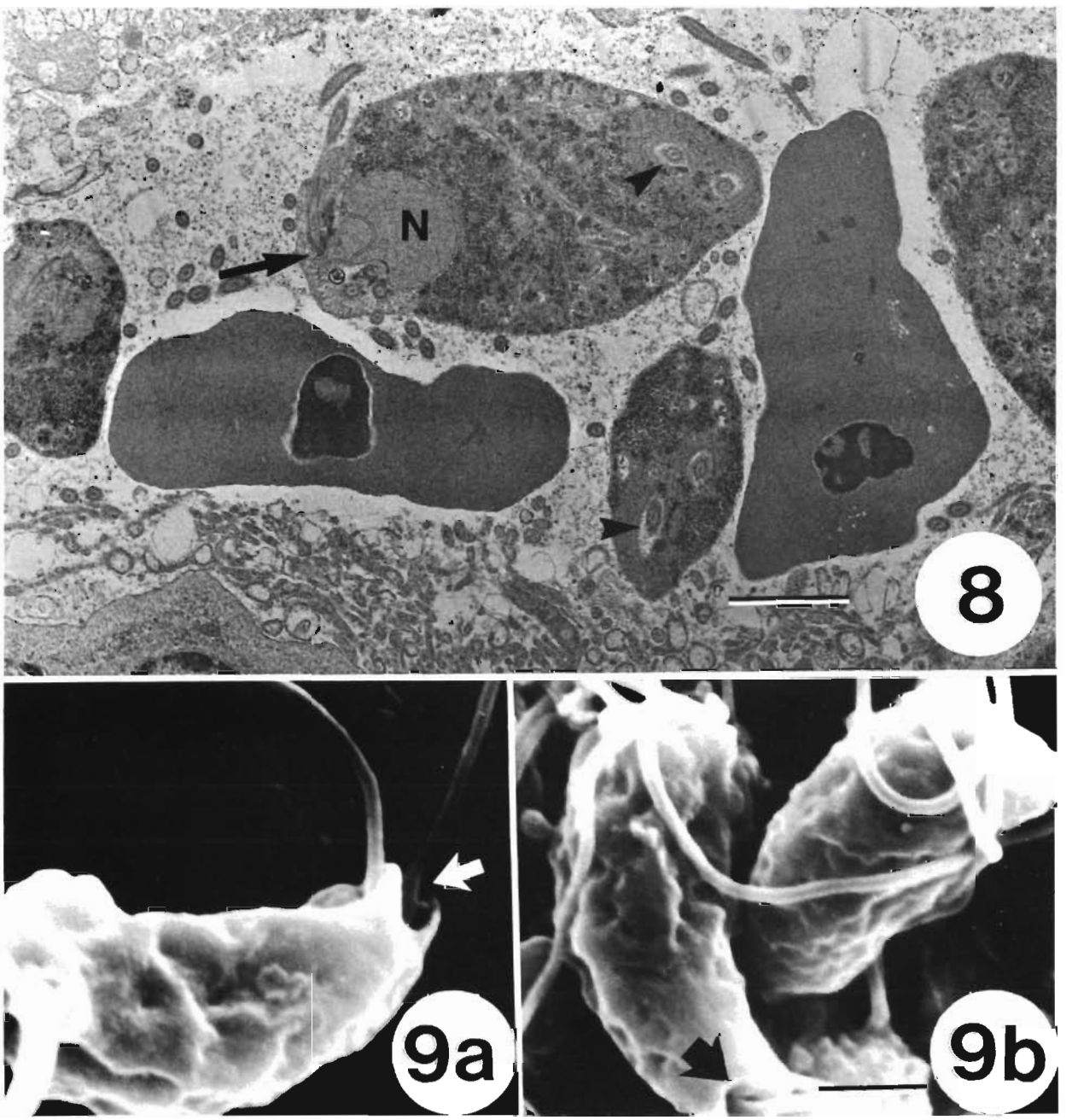
DISCUSSION

This systemic hexamitid infection was a serious disease at this netpen site. Essentially all the moribund fish that were examined from the 1990 lot in October 1991 exhibited massive numbers of the parasites in the blood, and the infection was not associated with detectable bacterial or viral infections. We, therefore, conclude that the parasitic septicemia described here was the primary cause of high mortality in the 1990 lot during fall and winter 1991. The prevalence of the infection was low in the 1991 lot and did not cause significant mortality. However, observation of the infection in moribund and dead fish from this group in late winter 1992 indicates that the disease may also eventually become a problem in this population as well.

Our transmission experiments demonstrated that the

infection and associated disease are easily transmitted in both fresh and sea water. In these experiments, previously healthy (and presumably immunocompetent) fish readily developed the infection, exhibited the typical pathological changes observed in the field and ultimately died. This suggests that, although the flagellate is morphologically indistinguishable from the relatively non-pathogenic *Hexamita salmonis* that infects the intestinal tract of salmonids in freshwater, it may represent a new, highly invasive strain of this species or perhaps a new species.

A similar disease was originally observed in seawater pen-reared Atlantic salmon at 2 sites in Norway (Mo et al. 1990), but now has been seen from at least 7 sites (T. Poppe, National Veterinary Institute, Oslo, Norway, pers. comm.). The infection appeared somewhat similar



Figs. 8 & 9. *Oncorhynchus tshawytscha*. Electron microscopy of the hexamitid flagellate from the liver of chinook. Fig. 8. Transmission electron microscopy. Arrow: medial-anterior kinetosomal complex; N: nucleus; arrowheads: recurrent flagella in flagellar pockets. Scale bar = 2 μm. Fig. 9. Scanning electron microscopy. Note funnel-shaped opening in flagellar pocket (arrows). Scale bar = 2 μm

to the chinook infection, except that the Atlantic salmon exhibited focal, granulomatous lesions. The flagellate from the chinook may represent a different strain or species from that causing the systemic infections in pen-reared Atlantic salmon in Norway (Mo et al. 1990) because we were unable to experimentally infect Atlantic salmon with the chinook parasite. This transmission experiment also suggests that the chinook parasite is different from *Hexamita salmonis* because the latter has been reported from Atlantic salmon (Kulda & Lom 1964).

The taxonomy of hexamitid parasites of fishes is not entirely clear, and it is possible that *Hexamita salmonis* should not be retained in the genus *Hexamita*. The flagella of *H. salmonis* arise anterior-medially (similar to *Spironucleus* and *Octomitus*) (Kulda & Lom 1964, Becker 1977, present study). In contrast, 1 criterion that separates *Hexamita* from other closely related hexamitid genera is the anterior-lateral location of the flagella in the former (Brugerolle 1974, 1975, Lee 1985, Woo & Poynton 1992). *Hexamita salmonis* was originally placed in the genus *Octomitus*. This genus is distinguished from *Hexamita* by the absence of flagellar pockets (Lee 1985).

Although we have demonstrated that the disease was transmissible in sea water, we were unable to determine if the chinook population at this netpen site originally contracted the infection in sea water or if they were subclinically infected when they were transferred to the netpens. Hexamitid flagellates, including *Hexamita* spp., have been reported from many marine fishes (Lavier 1936, Kulda & Lom 1964, Woo & Poynton 1992). The systemic infection was noted in the chinook after they had been in sea water for over a year. The younger year class at the farm (which had been in sea water for only 6 mo and came from the same freshwater hatchery) showed a much lower prevalence of the infection. These observations indicate that the parasite may be a marine organism that infects chinook salmon after they are transferred to sea water. The *in vitro* survivability test supports this hypothesis because the parasites survived longer in sea water than fresh water. Lastly, the freshwater hatchery that served as the source of the 1990 lot of chinook also supplied 8 other netpen sites in the Sechelt area in 1990. Examination of numerous moribund specimens at these sites by one of the authors (Ellis) failed to reveal the infection. This freshwater hatchery no longer rears chinook, so we were unable to examine pre-smolt chinook from the hatchery.

Controversy exists concerning the cyst and intracellular stages in *Hexamita salmonis*. Early reports described both of these stages by light microscopy (Moore 1922, Davis 1926), and cysts have been described for other species of *Hexamita* from fishes (Lavier 1936). More recent studies have failed to reveal these stages (Kulda & Lom 1964, Ferguson 1979) and Ferguson (1979) demonstrated by TEM that the presumed intracellular stages of *H. salmonis* in the gut are probably degenerated host cells.

We did not detect either cyst or intracellular stages in the present study, which suggests that the systemic infection is transmitted in fresh water and sea water directly from fish to fish by trophozoites. The *in vitro* survival experiments showed that the trophozoites survived poorly in sea water and that they were quickly killed in fresh water. This was probably due to an inability of the trophozoites to tolerate osmotic pressures that are significantly different from that of fish blood. The parasite appeared unaffected in mucus from skin scrapings. Possibly the flagellate is transmitted in mucus or fecal material, both of which could provide an environment that temporarily protects the organism from severe osmotic stress.

The gross and pathological changes observed in infected chinook were similar to those reported for other blood flagellate infections of fish. As observed in the present study, salmonids with heavy *Cryptobia salmositica* infections show abdominal distention with ascites, reno-splenomegaly and anemia (Woo 1987). The histologic changes in chinook with the hexamitid infection were similar to those seen in goldfish, *Carassius auratus* with *Trypanosoma danilewskyi* infection, in that both infections evoked hyperplasia of hemoblasts in the renal interstitium (Dyková & Lom 1979).

The anemia observed in salmonids with *Cryptobia salmositica* infections is due to hemolytic components from the parasite and formation of immune complexes on erythrocytes (Thomas & Woo 1988). Anemia in goldfish with *Trypanosoma danilewskyi* infections was caused by hemolysis and hemodilution due to increased blood volume (Nazrul Islam & Woo 1991). Chinook with the systemic hexamitid infection exhibited severe edema, serosanguinous ascitic fluid, visceral hemorrhages and blood clots. These changes suggest that the anemia may have been due, at least in part, to hemodilution and hemorrhage.

Numerous therapeutic agents have been used to treat intestinal *Hexamita salmonis* infections in salmonids (Yasutake et al. 1961, McElwain & Post 1968, Hoffman & Meyer 1974, Becker 1977). Compounds that are absorbed through the intestinal tract and occur in high concentrations in the blood (e.g. Fumagillin DCH) may prove useful for treating the systemic infection. In addition, identification of the source of the infection would be helpful for implementing effective control strategies or prophylactic treatments.

Ferguson (1979) described the ultrastructure of *Hexamita salmonis* and associated ultrastructural changes in gut infections of rainbow trout *Oncorhynchus mykiss*

by TEM, and Poynton & Morrison (1990) and Ferguson (1979) described surface features of the parasite by SEM. However, more detailed ultrastructural studies (by both SEM and TEM) of *H. salmonis*, the parasite from the blood of chinook, and related hexamitids are needed to determine the precise taxonomy of these organisms. A thorough ultrastructural comparison of the parasite from the chinook blood with *H. salmonis* from the gut of freshwater salmonids is planned. In addition, further studies on the host susceptibility of the parasite are under way. The hexamitid parasite has been deposited to the American Type Culture Collection, Rockville, Maryland, USA, ATCC 50329 and 50330.

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