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The Development of the PKX Myxosporean, the Causative Agent of Proliferative Kidney Disease, in Rainbow Trout Salmo gairdneri Richardson

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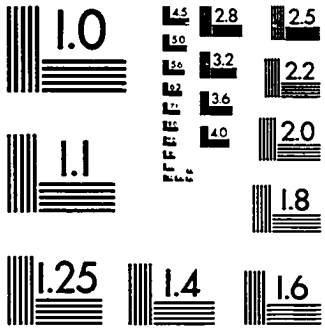

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THE DEVELOPMENT OF THE PKX MYXOSPOREAN, THE CAUSATIVE AGENT OF PROLIFERATIVE KIDNEY DISEASE, IN RAINBOW TROUT SALMO GAIRDNERI RICHARDSON

University of California, Davis

PH.D. 1985

The Development of the PKX Myxosporean, the Causative Agent of Proliferative Kidney Disease, in Rainbow Trout Salmo gairdneri Richardson

By

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DISSERTATION

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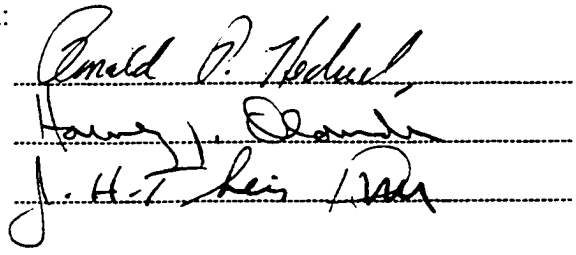

Committee in Charge

1985

The Development of the PKX Myxosporean, the Causative Agent of Proliferative Kidney Disease, in Rainbow Trout Salmo gairdneri Richardson.

Abstract

The endogenous development of PKX, the causative agent of proliferative kidney disease, is described in rainbow trout, Salmo gairdneri, by light and electron microscopy. Parasites multiplied by endogeny, binary fission and possibly plasmotomy in the renal interstitium, and elicited an interstitial nephritis. As the disease progressed parasites were found in the lumens of the tubules, and these represented daughter cells released from PKX parasites that had migrated to the tubules. Internal sporoblasts formed within these daughter cells (enveloping cells). The internal sporoblasts contained up to six daughter cells and some sporoblasts organized into spores with two spherical polar capsules. Although the spores persisted for several months after the interstitial PKX and associated inflammation had subsided, they did not complete their development as indicated by the fact that they remained within the enveloping cell and did not form valves.

The relationship of this spore to the vegetative stages was studied further by a transmission experiment. The myxosporean forms were only observed in the kidney tubules, while typical PKX also occurred in the blood and spleen. Parasite-free fish injected with the blood and spleen of infected fish developed both forms of the parasite, which further substantiates that PKX is the prespore form of the intraluminal myxosporean.

Because only incomplete spores were observed, the more precise taxonomic status of PKX was not determined. However, intraluminal stages of PKX show similarities to the genera Sphaerospora, Mitraspora and Parvicapsula. Furthermore, interstitial stages of PKX resemble the early stages of Sphaerospora.

The incomplete spore development and severe inflammatory response to PKX indicates that salmonids may be abnormal hosts. Fish were immunosuppressed with cortisol implants, and they exhibited greater densities of interstitial and intraluminal PKX but less interstitial hypercellularity. However, spores did not develop further than those observed in natural epizootics. This study supports the hypothesis that PKX is an early form of a myxosporean and that salmonids are abnormal hosts. A non-salmonid fish may be the primary host for PKX in which sporulation is completed.

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Introduction

Proliferative kidney disease (PKD) has occurred in several hatcheries in Europe and North America and is now considered a serious obstacle to the successful production of certain salmonid fishes. The disease has been reported most often in rainbow trout, Salmo gairdneri Richardson and mortalities may reach 90%, but usually remain below 30-50% (Clifton-Hadley et al. 1984).

Even in the absence of high mortality, morbidity may be 100% and fish with PKD exhibit poor tolerance to stress, decreased food conversion and greater susceptability to secondary infections (Seagrave and Bucke 1979; Seagrave et al. 1981). The disease inflicts further economic burdens on production facilities because husbandry practices must be modified and the planting of exposed fishes is often restricted by regulatory agencies.

Proliferative kidney disease is caused by an unclassified protozoan (PKX) (Seagrave et al. 1980a). Plehn (1924) described a disease in salmonids that she believed was caused by an amoeba in the kidney which was most likely PKD. Because Ghittino et al. (1977) and Ferguson and Needham (1978) observed PKX to form pseudopodia, they considered it an amoeba. Although no spores were observed, Seagrave et al. (1980a) proposed that the organism might be a haplosporidan (phylum Acetospora). Division by internal cleavage, the presence of multivesicular bodies, and

electron dense cytoplasmic inclusions reminiscent of the haplosporosomes of the invertebrate parasite Marteilia were cited as common features with PKX.

Hedrick et al. (1984) and Kent and Hedrick (1985) observed myxosporean trophozoites and developing spores in the renal tubules of PKD infected fish, and they suspected that they were later stages of PKX and that PKX belongs to the phylum Myxozoa. Although the life cycles of the phyla Myxozoa and Acetospora are poorly understood, they share similar developmental stages and the higher taxonomy of these parasites is in confusion. A more thorough understanding of the life cycle and taxonomy of PKX will be ultimately required to control the disease.

The objectives of this study were to describe the sequential development of PKX in rainbow trout, and to elucidate the relationship of this protozoan with myxosporean sporogonic stages described by Hedrick et al. (1984) and Kent and Hedrick (1985). Seagrave et al. (1980a) proposed that salmonids may be abnormal hosts for PKX. This would account for the severe inflammation associated with clinically affected fish, which is unusual for most myxosporean infections (Dyková and Lom 1978). Since the inflammatory response may be a major factor in preventing parasites from completing their development in abnormal hosts, the effects of immunosuppression on the inflammation that is commonly associated with PKD and the development of the parasite were investigated.

Literature Review

Pathology. Proliferative kidney disease (PKD) of cultured salmonid fishes is characterized by chronic inflammation of the kidney and other visceral organs. Affected fish typically exhibit lateral body swelling due to kidney hypertrophy and abdominal distention from ascitic fluid accumulation due to kidney failure (Ferguson and Needham 1978; Clifton-Hadley et al. 1984). Bilateral exophthalmia, hypertrophy of the spleen and anemia as evidenced by pallor of the gills, are also common clinical signs (Ghittino et al. 1977; Ferguson and Needham 1978; Hedrick et al. 1984). The anemia associated with PKD was originally described as hypoplastic (Robertson 1978), but a thorough investigation by Hoffman and Lommel (1984) indicated that it is instead a chronic hemolytic anemia caused possibly by toxins from the parasite. Ferguson and Needham (1978) reported PCV values of 11% compared to 28-31% for uninfected fish. Hoffman and Lommel (1984) also reported lower mean PCV values in PKD affected fish (28% versus 40% in uninfected fish). They also showed no leucocytosis in PKD affected fish. Additionally, hypoproteinemia is a characteristic of PKD.

Sections of infected tissues reveal the PKX protozoan primarily in the kidney interstitium, associated with interstitial nephritis and tubular atrophy (Ferguson and Needham 1978; Clifton-Hadley et al. 1984; Smith et al. 1984). The kidney is an important organ for hemopoiesis in fish (Ellis et al. 1978), and the initial response to the parasite is

proliferation of hemopoietic cells (Clifton-Hadley et al. 1985). However, the major tissue response in the kidney is interstitial hypercellularity due to infiltration and proliferation of mononuclear cells, presumably macrophages, which form "whorls" around the parasite (Ferguson and Needham 1978). Lymphocytes are also closely associated with PKX, and Clifton-Hadley et al. (1985) suggested that this may indicate that an active immune response occurs. This was further supported by Klontz et al. (1985) and Olesen and Jorgensen (1985) who reported substantial increases in the beta serum fraction of PKD affected fish. If fish survive PKD, the interstitial hypercellularity will subside in 12 to 20 wk, and the kidneys of recovered fish show little if any signs of previous infection (Clifton-Hadley et al. 1985).

In heavily infected fish, PKX is often found in the spleen, gut, pancreas, gills, liver and muscle, where it also can invoke a granulomatous response (Ferguson and Needham 1978; Smith et al. 1984). The PKX parasite most likely reaches these organs via the circulatory system and it may adhere to the vessel walls, provoking a necrotizing vasculitis (Smith et al. 1984).

Factors affecting the severity of PKD. Epizootics of PKD vary markedly in severity (Clifton-Hadley et al. 1984). Mortalities have reached 95% in affected fishes (Seagrave and Bucke 1979; Hedrick et al. 1984). However even with high intensities of PKX and significant morbitity, few mortalities may result (Clifton-Hadley et al. 1984; Hedrick et al. 1985).

This discrepancy in mortalities between epizootics may be due to factors other than prevalence and intensity of the parasite. Affected fish are prone to respiratory distress due to chronic anemia (Seagrave and Bucke 1979), and the latter is probably the primary cause of death in PKD (Clifton-Hadley et al. 1984). Considerable losses may occur if fish are handled or if oxygen levels are low in the water (Clifton-Hadley et al. 1984).

Additionally, concurrent infections with other pathogens such as Ichthyophthirius, Flexibacter, Costia, infectious pancreatic necrosis virus, and infectious hematopoietic necrosis virus have been associated with PKD and they may contribute to mortality (Hoffman and Dangschat 1981; Smith et al. 1984; Busch 1985; Hedrick et al. 1985). Roberts (1978) and Ferguson and Needham (1978) considered soft acid water to predispose fish to the effects of PKD, but the disease has also occurred in hard akaline waters (Scott 1979; Hedrick et al. 1984).

Proliferative kidney disease usually occurs during the summer months when water temperatures are greatest, and most reports have been from fish held above 15 C (Clifton-Hadley et al. 1984). However, Hedrick et al. (1984) reported a severe epizootic in Pacific salmon and steelhead trout, S. gairdneri at 12-14 C, and Rafferty et al. (1985) found that rainbow trout injected with PKX and then held at 7 and 11 C developed clinical PKD.

The disease occurs most often in fish under one year of age (Roberts and Shepard 1974; Ghittino et al. 1977; Ferguson and Needham 1978;

Hedrick et al. 1984; Hedrick et al. 1985), but older fish have been affected (Ferguson and Ball 1979; Hoffman and Dangschat 1981; D'Silva et al. 1984). Although a group of small fish (avg. wt. 7.5g) were 100% infected at a hatchery in California, the highest mortality and most severe response to the parasite occurred in larger fish (avg. wt. 45g) (Hedrick et al. 1985). Surviving fish are resistant to reinfection the following year (Ferguson and Ball 1979; Hedrick et al. 1985), and natural immunity in certain stocks of Atlantic salmon, Salmo salar L. has been reported by Ellis et al. (1982).

Hosts and Geographical Locations. Plehn (1924) described an amoeboid parasite in brook trout, Salvelinus fontinalis (Mitchell) which was most likely PKX. Schäperclaus (1954; as cited from Clifton-Hadley et al. 1984) described a condition similar to PKD in rainbow trout but the etiology was confused by chronic infections of viral hemorrhagic septicemia. However, proliferative kidney disease and the PKX protozoan were not well described until the 1970's (Roberts and Shepard 1974; Ghittino et al. 1977; Ferguson and Adair 1977; O'Brien and McArdle 1977; Ferguson and Needham 1978). Now it is considered one of the most important diseases of cultured salmonid fishes in Europe and has occurred in Italy, France, Germany, England, Wales, Scotland, Ireland, Sweden (Clifton-Hadley et al. 1984) and Denmark (Olesen et al. 1983). In 1981, the first report in North America was made by Smith et al. (1982) who found the disease in cultured rainbow trout. Subsequently the disease

river drainages in British Columbia, Canada (Hoskins 1985) and in California, USA (Hedrick et al. 1984). However, after reviewing histological slides, California Department of Fish and Game biologists determined that the parasite has been present in California at the American River hatchery since 1966 and was responsible for a conditon they previously called "lupus" (Hedrick et al. 1985).

Most cases of PKD have been reported from rainbow trout (Clifton-Hadley et al. 1984), but other cultured salmonids have been infected. These include brook trout, (Plehn 1924), brown trout, Salmo trutta L. (Roberts 1978; Seagrave et al. 1981; Ellis et al. 1985), king salmon, Oncorhynchus tshawytscha (Walbaum) and silver salmon, O. kisutch (Walbaum) (Hedrick et al. 1984) and Atlantic salmon, (Ellis et al 1982; Ellis et al. 1985). In an epizootic in California, the disease affected king salmon more severely than steelhead trout or silver salmon held under the same conditions (Hedrick et al. 1984). The disease is primarly a problem of cultured fishes, but the parasite has been detected in wild rainbow trout (Olesen et al. 1983), char, Salvelinus alpinis (L.) (Bucke et al. 1985) and grayling, Thymallus thymallus (L.) (Seagrave et al. 1981). The only non-salmonid that has been shown to harbor PKX is the pike, Esox lucius (L.) (Seagrave et al. 1981).

Etiological Agent. Although the PKX parasite has been described by light and electron microscopy (Ferguson and Needham 1978; Seagrave et al. 1980 a,b; Smith et al. 1984), its taxonomnic status remains unresolved.

In paraffin sections the parasites are 10-20 μm in diameter and are amoeboid with pseudopodia (Ferguson and Needham 1978). The PKX parasite is usually multinucleated and the nuclei have large prominent endosomes with radiating chromatin patterns. The cytoplasm is eosinophilic and has periodic acid Schiff (PAS) positive granules (Ferguson and Needham 1978; Hedrick et al. 1984). Internal daughter cells are frequently observed (Ferguson and Needham 1978).

Ferguson and Needham (1978), Seagrave et al. (1980 a,b) and Smith et al. (1984) described a similar ultrastructual anatomy for PKX. A prominent feature is the presence of numerous electron dense cytoplasmic inclusions, referred to as "haplosporosomes" by Seagrave et al. (1980a). They are 0.14 -0.20 μm in diameter, have a characteristic electron lucent bar and are often associated with the plasmalemma. A well developed endoplasmic reticulum, numerous multivesicular and lipoid bodies, and internal daughter cells bound by double membranes have been described consistently in PKX. The parasite divides by endogeny, and tertiary as well as secondary daughter cells have often been observed. The daughter cells have a similar ultrastructure to the surrounding primary mother cell, except they do not contain haplosporosomes.

A parasite that was most likely PKX was described as an amoeba by Plehn (1924), and Ghittino et al. (1977) and Ferguson and Needham (1978) suggested that PKX may be an amoeba because it formed pseudopodia. Ghittino et al. (1977) reported *in vitro* culture of an amoeba that they

proposed was PKX, but subsequent ultrastructure examination by Ferguson et al. (1978) revealed that it was a different organism.

Although no spores were observed, Seagrave et al. (1980a,b) proposed that PKX might be a haplosporidan (phylum Acetospora), which are parasites of crustaceans and molluscs and have not been reported to infect vertebrates (Ginsburger-Vogel 1979; Perkins 1979). Division by endogeny, the presence of multivesicular bodies, and electron dense inclusions reminiscent of the haplosporosomes of the oyster pathogen Marteilia were cited as common features with PKX. Ferguson and Needham (1978) and Seagrave et al. (1980a,b) did not exclude the possibility that PKX may belong to the phylum Myxozoa. Members of this phylum, which are ubiquitous parasites of fish, also divide by endogeny, have amoeboid trophozoites and have structures similar to haplosporosomes (Current and Janovy 1977; Current et al. 1979; Lom et al. 1982; Desser et al. 1983).

Myxosporean trophozoites and developing spores in the kidney tubules of PKX infected fish were reported by Hedrick et al. (1984) and Kent and Hedrick (1985), and they proposed that these were later stages of PKX and that the parasite belongs most appropriately to the phylum Myxozoa. Those authors compared the myxosporean forms with members of the family Sphaerosporidae, certain of which are parasites of cyprinid fishes and sporulate in the kidney tubules (Ahmed 1973; Hamilton 1980; Molnár 1980; Dyková and Lom 1982; Lom et al. 1982; Lom et al. 1983; Hoffman 1984; Lom et al. 1985). Most myxosporeans form multinucleated syncytia, called plasmodia, which contain the sporoblasts (Mitchell 1977); but

Sphaerospora spp. form "pseudoplasmodia". The pseudoplasmodium is homologous to the sporoblast of most myxosporeans in which the sporogonic cells are surrounded by a pericyte or enveloping cell (Lom et al. 1982).

As with PKX, the earliest stages of Sphaerospora may occur in the blood. An unidentified blood protozoan of common carp, Cyprinus carpio L. was first reported by Csaba (1976), and Buscek and Csaba (1982) proposed that it may belong to the Myxozoa. Lom et al. (1983) also concluded that this parasite, designated UBO or Csaba's blood protozoan, is a myxosporean. They cited the following as similar characteristics shared by UBO and the Myxozoa: lack of centrioles, bundles of microtubules associated with the nuclei, numerous free ribosomes in the secondary cells, pericyte nature of the primary cells, proliferation by disintergration of the primary cell, and no fixed rigidity in the vegetative development. Those authors reported that UBO most likely represented early stages of Sphaerospora renicola Dyková and Lom because a strong correlation between the presence of UBO in the blood and Sphaerospora in the kidney tubules was observed in carp.

Other cyprinid fishes such as tench, Tinca tinca (L.), roach, Rutilus rutilus (L.) and gudgeon, Gobio gobio (L.) are infected with both blood protozoans similar to UBO and Sphaerospora spp. (Lom et al. 1985). Another protozoan similar to UBO was described by Kovács-Gayer et al. (1982; cited from Lom et al. 1983) from the swimbladder of carp and this parasite may be an early stage of S. angulata (Csaba et al. 1984; Molnár 1984). Sphaerospora has been detected in brown trout from PKD

enzootic waters (Ferguson 1984), and Lom et al. (1985) detected Sphaerospora trophozoites and possible early blood forms in brown trout from Czechoslovakia. They suggested that presporogonic stages similar to UBO may exist for all Sphaerospora spp. The swimbladder protozoan and UBO are amoeboid and multiply by internal cleavage, and in these respects are identical to PKX (Csaba et al. 1984).

Transmission. Successful transmission of PKX by exposure to water where the disease is enzootic (Ferguson and Ball 1979; Ferguson 1981; D'Silva et al. 1984) or by intraperitoneal injection of infected tissue (Clifton-Hadley et al. 1984; D'Silva et al. 1984; Rafferty, et al. 1985) has been achieved, but direct transmission by cohabitation with infected fish in aquaria or feeding infected tissue has been unsuccessful (Ferguson and Ball 1979; D'Silva et al. 1984). The possibility that an intermediate host is involved in the life cycle has been proposed by de Kinkelin and Gerard (1977) and Seagrave et al. (1981).

The incubation period is approximately four weeks when fish are infected by injection or natural exposure, and clinical signs follow shortly thereafter (Ferguson and Ball 1979; Clifton-Hadley et al. 1985). At lower temperatures the incubation period is extended, but the disease is still expressed (Rafferty et al. 1985).

Ghittino et al. (1977) proposed that ingestion of the infective stage may be the route of entry, but this remains to be verified. The parasite is observed in the blood vessels and it most likely reaches the

kidney via the circulatory system (Ferguson and Needham 1978; Clifton-Hadley et al. 1984). Ferguson and Needham (1978) observed parasites in the kidney tubule lumen, and they suggested that this may be the mode of exit.

Control. Antibacterial and antiprotozoan compounds have failed to control PKD (Ghittino et al. 1977; Ferguson and Ball 1979; Bucke et al. 1981). Therefore, changes in husbandry practices are used to reduce the effects of the disease. Because PKD infected fish are extremely susceptible to stress, especially low oxygen, handling should be kept to a minimum, and low densities of fish with good water flow should be maintained during epizootics (Seagrave and Bucke 1979; Bucke et al. 1981). Ferguson and Ball (1979) found that lower feeding levels and introduction of fish later in the summer reduced the affects of PKD. When fish were introduced at this time, they still became infected, but the clinical signs were reduced and they recovered faster due to a drop in water temperature with the onset of fall. Most of these fish were also protected against reinfection the following year; they exhibited 20% morbidity and 1% mortality. However, Bucke et al. (1981) did not find this control measure effective because survivors exhibited a marked reduction in growth.

Immunosuppression. Seagrave et al. (1980a) proposed that salmonids may be abnormal hosts for PKX, which would account for the severe

inflammatory response and lack of complete development of the parasite (Dykova and Lom 1978). It is well documented that higher vertebrates can be abnormal hosts for parasites of many taxa and infections by their own parasites will intensify when they are immunosuppressed (Dale and Petersdorf 1973; Chapman 1974; Ali-kan and Seemeyer 1975; Bach 1976; Gannon 1980). As with higher vertebrates, stress increases the susceptibility of fish to disease (Wedemeyer and McLeay 1981), and corticosteroid levels increase in fish stressed by handling, disease, anesthesia and pollution (Donaldson 1981). High corticosteroid levels cause lymphopenia in fish (McLeay 1973; Anderson et al. 1982) and it is presumed that this is due to direct lymphocytolytic actions of the drugs (McLeay 1973). It is suspected that the effects of corticosteroids on macrophages of fish is similar to that in mammals, i.e. causing monocytopenia, decreased lysosome to phagosome fusion and decreased metabolic activity (Ellis 1981). Therefore, high blood corticosteriod levels are a primary factor in the increased susceptibility of stressed fish to disease (Wedemeyer 1970).

This has been supported by experimental evidence. Robertson et al. (1963) induced high cortisol blood levels in rainbow trout by implanting cortisol mixed with cholesterol at approximately 0.47 mg cortisol/g fish. This allowed a massive proliferation of the ciliate parasite Ichthyophthirius and fish died in 5 to 9 weeks. Pickering and Duston (1983) induced sustained high cortisol levels in brown trout with implants of cortisol mixed with cocoa butter at 0.016 - 0.16 mg

cortisol/g fish, and they succumbed to Aeromonas salmonicida infections at the higher levels. These authors also demonstrated that prolonged oral administration of cortisol increased the susceptibility of brown trout to the opportunistic fungus Saprolegnia.

In rainbow trout, lymphocytes represent 90 - 97% of circulating leukocytes (Ellis et al. 1978), and lymphopenia occurs in stressed and corticosteroid treated fish (McLeay 1973; Ellis 1981; Anderson et al. 1982). The number of circulating leukocytes reflects the fish's reaction to stress (McLeay and Gordon 1977) and can be used as an indication of immunosuppression. To facilitate the estimation of leukocytes a "leucocrit" test was developed by McLeay and Gordon (1977). This test is based on the determination of the volume of packed leukocytes (buffy layer) in relation to total blood volume. Blood is collected in heparinized capillary tubes, centrifuged and the buffy layer is measured using a microscope with a calibrated ocular micrometer.

Although immunosuppressive drugs have not been used to induce fish parasites to develop in abnormal fish hosts, they have been used to investigate host-parasite relationships in higher vertebrates. Examples include studies with coccidia (phylum Apicomplexa), which are typically very host specific. Todd and Lepp (1971) reported the completion of the endogenous life cycle of a mouse eimerian in rats following dexamethazone treatment, and McLoughlin (1969) induced Eimeria meleagrimitis of turkeys to complete development in dexamethazone treated chickens. Further development of helminths in abnormal hosts treated with

corticosteroids has also occurred. Wong (1974) recovered adult filarial worms (Dirofilaria immitis) from prednisolone and triamcinolone treated monkeys (Macaca sp.) and Hashiguchi and Hirai (1977) reported enhanced growth and maturation of the fluke Paragonimus miyazakii in rats treated with immunosuppressants. Therefore, immunosuppression by administration of exogenous corticosteroids is a useful technique to enhance the development of parasites in abnormal hosts.

Materials and Methods

Sequential Development and Prevalence of PKX

A group of rainbow trout under one year of age (Eagle Lake, California strain) were introduced into water containing the infective stage of the PKX parasite at the American River hatchery, Rancho Cordova, California, USA on 16 August 1984. Approximately 500 of these fish were transferred to the fish pathology wet laboratory at the University of California (U.C.), Davis on 9 September 1984 and maintained in PKX-free well water at 15 - 18 C. Samples of 15-30 fish were taken at different times from the population at the hatchery and from the fish at U.C. Davis until May 1985 as indicated in Figures 1 and 2.

To determine the morphology of PKX, preparations of fresh kidneys were squashed between a glass slide and cover slip (wet mounts), and then examined by bright field and phase contrast microscopy. For histological examinations, tissues were fixed in Davidson's solution (Humason 1979), and then embedded in paraffin, sectioned at 5 μm and stained with either hematoxylin and eosin (H&E), periodic acid Schiff's (PAS) or Price's Giemsa as described by Luna (1968) and Humason (1979). Fish from the sequential samples taken at the American River hatchery and U.C. Davis were examined for the prevalence of interstitial PKX, associated pathology and intraluminal myxosporean stages using paraffin sections of the kidneys. Kidney tissue samples for electron microscopy were fixed in 3% (v/v) glutaraldehyde in phosphate buffer as described by Bullock

(1978). These specimens were post fixed in 1% OsO_4, embedded in Epon, sectioned, stained with lead citrate and uranyl acetate and observed with a Phillips EM 400 transmission electron microscope. The developmental stages of PKX described here were observed in all epizootics of PKD in California in 1983 and 1984, and fish from several locations were used for the light and electron microscopical descriptions.

Blood and Spleen Transmission of PKX

The myxosporean trophozoites and spores that occur in PKD infected fish are found only in the lumens of the kidney tubules and never in the blood or spleen where typical PKX is often observed. To determine if fish receiving blood and spleen tissues of infected fish become infected with both typical PKX and the intraluminal myxosporeans, the following experiment was conducted.

The blood from 20 rainbow trout with PKD was collected in heparinized capillary tubes and 0.2 ml/fish was injected into the peritoneal cavity of 20 PKX-free steelhead trout with an average weight of 11 g. The steelhead trout were obtained from the Warm Springs hatchery, Sonoma County, California, where PKD has never been observed. The spleens from two infected trout were disrupted by forcing them through a wire sieve with Minimal Essential Medium (MEM) and injected into the peritoneal cavity of 20 additional steelhead trout. A control group of 20 steelhead that received 0.2 ml of MEM was also included. Wet

mounts and paraffin sections of the kidneys were then examined at 8 to 12 wk post injection to evaluate the presence of both interstitial PKX and the intraluminal myxosporeans.

Cortisol Treatment Studies

Fish treated with cortisol implants were used to test the effects of immunosuppression on the development of PKX and the associated inflammatory response. The dosage was determined by preliminary studies in this laboratory and by those of others (Robertson et al. 1963; Hill and Fromm 1968; Pickering and Duston 1983; C. Schreck, Oregon State University, OR., USA, pers.comm.). These studies indicated that cortisol implants administered by intraperitoneal injection at 0.5 mg/g fish would induce elevated blood cortisol levels and chronic immunosuppression. In addition, preliminary studies showed that cocoa butter implants without cortisol did not induce immunosuppression. Plasma cortisol levels, leucocrits and cumulative mortalities to opportunistic pathogens were all used as indicators of immunosuppression.

Exposure to PKX. Approximately 150,000 rainbow trout fingerlings were transferred to the American River hatchery from the Moccasin Creek hatchery, Sonora County, California on 8 December 1984. It was determined that the infectious stage of PKX was not present at the American River hatchery at this time (Hedrick et al. 1985), and the Moccasin Creek hatchery is known to be free of the parasite. The fish were examined by making histological sections of kidney tissues weekly

beginning April 1985 to detect the first appearance of PKX. The parasite was first detected in this population on 15 June 1985. The parasite is not detected until approximately 4 wk after exposure to infectious water (D'Silva et al. 1984; Clifton-Hadley et al. 1985), which indicates that the fish in the present study were first exposed to PKX in mid-May. Three hundred of these fish (avg. wt. 43 g) were then transferred to the U.C. Davis laboratory on 18 June 1985. Rainbow trout (avg. wt. 46 g) from the Moccasin Creek hatchery were transferred directly to U.C. Davis in March 1985 to be used as uninfected controls.

Cortisol Implantation. After three days acclimation (21 June 1985) fish were given cortisol implants. Cortisol was mixed with cocoa butter (liquefied by heating to 50 C) to a final concentration of 125 mg/ml. It was maintained in this state before injection by heating the solution and glass syringes on a hot plate at 50 C. Eighty PKX exposed fish and 80 controls were anesthetized with MS-222 (tricane methanesulfonate) at 12 mg/L, weighed and injected with 0.004 ml cortisol solution/g fish. This resulted in an implant of 0.5 mg cortisol/g fish. The remaining fish (non-immunosuppressed) were anesthetized and then injected with 0.004 ml MEM/g fish.

The four treatments (80 fish in each) were as follows: exposed to PKX and cortisol treated (PKX+C+), PKX-free and cortisol treated (PKX-C+), exposed to PKX and not cortisol treated (PKX+C-), and PKX-free and not cortisol treated (PKX-C-). All groups were maintained in 100 L

aquaria at a density of 20 fish/aquarium. The temperature was held at 16-17 C, except for on days 23 and 24 post injection when the temperature was 20 C due to failure of the chilling system. Fish were fed daily to satiation with a commercial dry pellet. To control external parasites, all fish were treated with flushes of malachite green at 1 ppm and formalin at 100 ppm three times weekly beginning on the third week of the experiment and thereafter until the end of the study. Seventeen fish from each group were examined at 4 wk post injection of the cortisol implants (PI). At 8 wk PI, 15 fish from PKX-C+ and 20 each from the other groups were examined. Fish were anesthetized with MS-222, the tail was severed at the caudal peduncle and blood was collected in heparinized capillary tubes for leucocrit determination as described by McLeay and Gordon (1977). Blood was also collected in 5 ml heparinized tubes for plasma cortisol analysis. The cortisol values were determined using an enzyme immunoassay (EIA) technique developed by Munro and Stabenfeldt (1983) modified for testing cortisol.

Two sagittal sections from the kidney of each fish were examined. They were fixed in Davidson's solution, sectioned at 7 μm and stained with H&E. Sections were taken from two different areas of the block separated by at least 1 mm. Typical interstitial PKX parasites and the number of tubules with the intraluminal forms were counted from 20 fields at a magnification of 400X (an area equivalent to 14.7 mm^2). The number of tubules/mm^2 was also determined and used to quantify the degree of interstitial proliferation and inflammation.

Analysis of variance (ANOVA), Fishers's least significant difference and Student's t tests ($p<0.01$) were used to analyze the leucocrit, plasma cortisol, tubule and PKX density data and these results are reported in the appendix.

Results

Sequential Development of PKX

Temporal Prevalence. The PKX parasite was first detected in one of 15 fish examined 3 wk after introduction to the American River hatchery (Figure 1). Parasites were easily detected the following week and reached a peak prevalence of 83% at 7 wk. Associated interstitial inflammation was evident at 5 wk, was most prominent between 8 and 11 wk, and reached a peak prevalence of 60%. The PKX parasite in the epithelium of the tubules and intraluminal sporogonic stages were first detected at 7 wk, and the latter reached a prevalence of 60% at 11 wk. The intensity of infection by the intraluminal forms steadily diminished after approximately 15 wk. However, the prevalence of these forms ranged from 38 to 67% after wk 10 and they persisted for at least 12 wk after interstitial PKX and associated lesions were not detected (Figure 1).

The sequential development of PKX in the fish transferred to the U.C. Davis laboratory was similar to that observed in the fish held at the hatchery (Figure 2). The prevalence of interstitial PKX and associated lesions decreased and intraluminal forms increased as the water temperature at the hatchery declined (Figure 1). However, the temperature at U.C. Davis was above 15 C throughout the study, and the intraluminal forms became prevalent in these fish as the disease progressed (Figure 2).

Figure 1. Sequential prevalence of interstitial PKX, intraluminal sporogonic forms and interstitial inflammation in the kidneys of rainbow trout, Salmo gairdneri, maintained at the American River hatchery. Samples of 15-30 fish were taken periodically from August 1984 through April 1985.

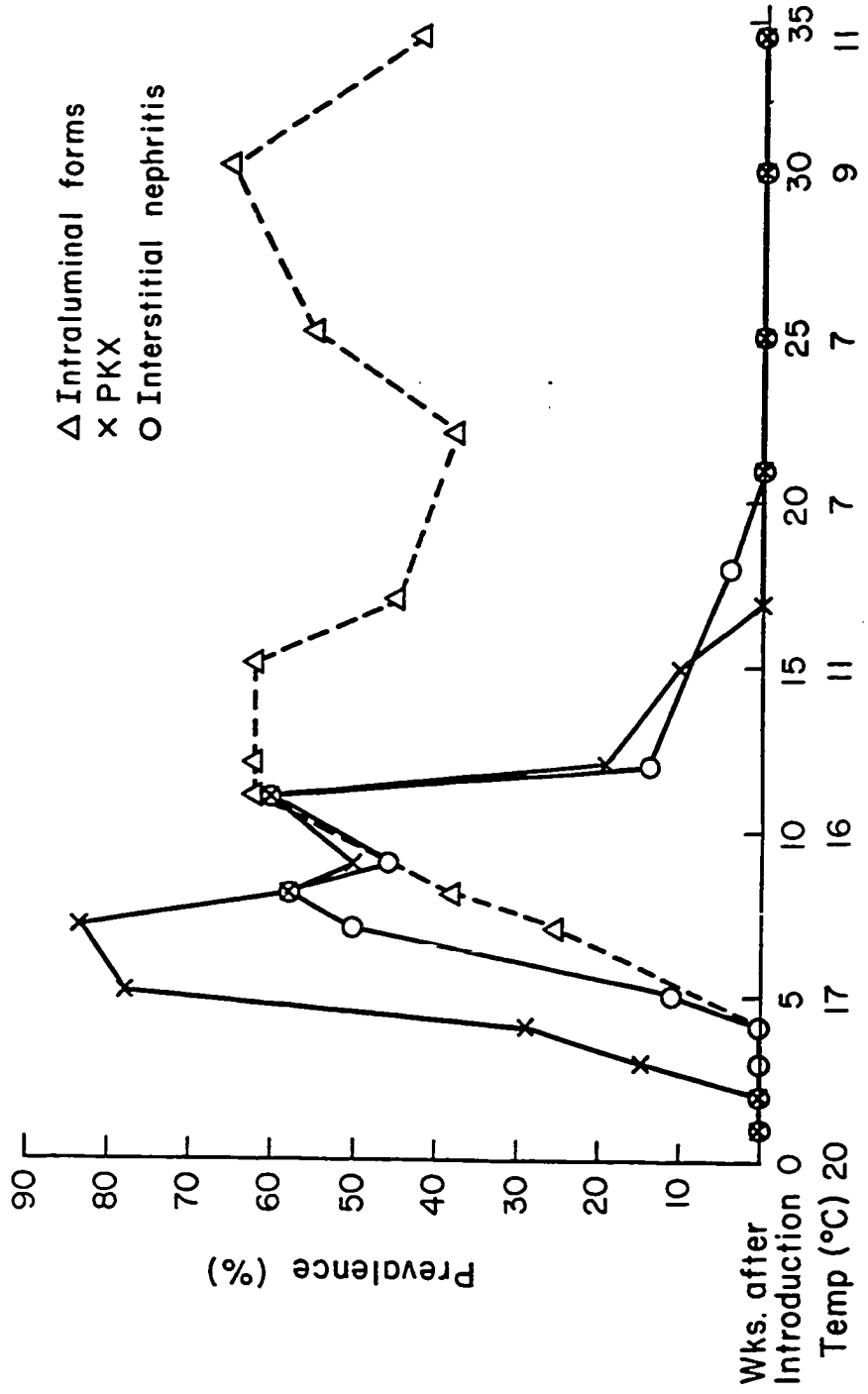
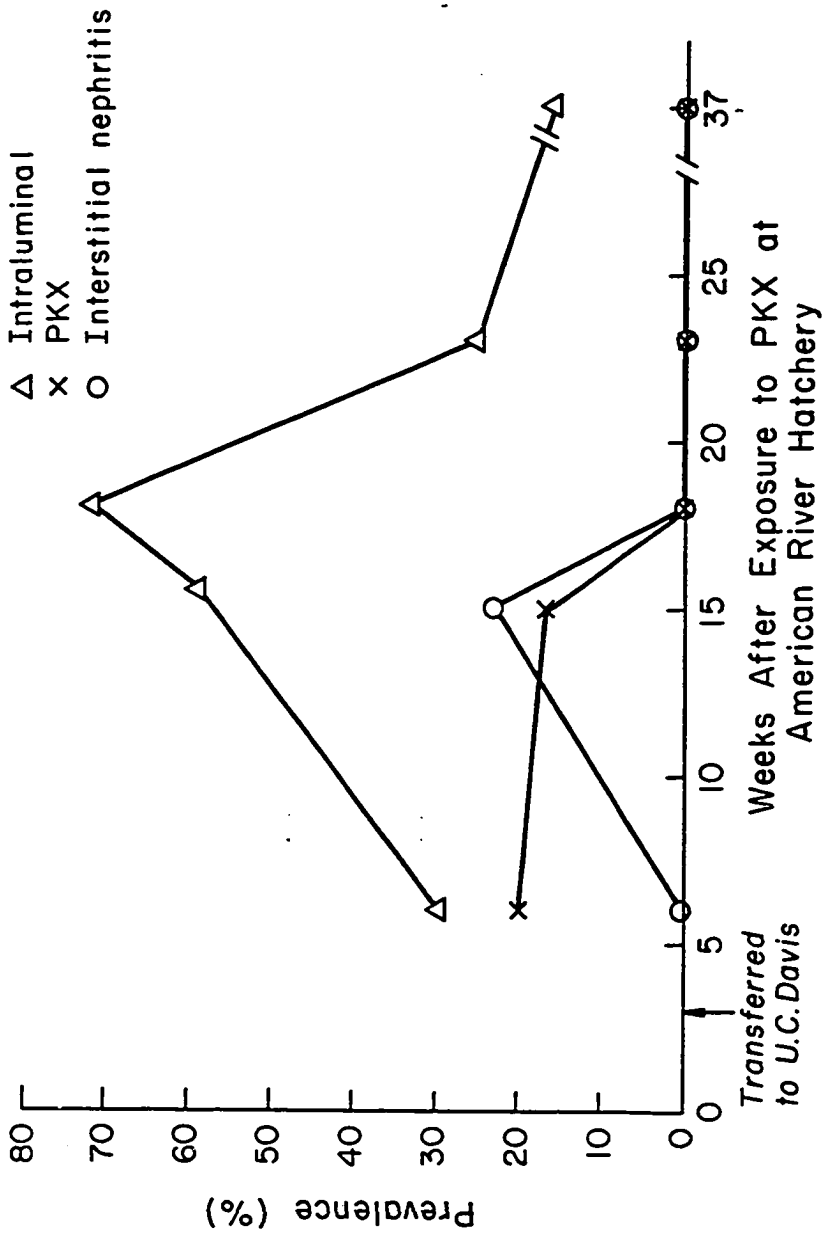


Figure 2. Sequential prevalence of interstitial PKX, intraluminal sporogonic forms and interstitial inflammation in the kidneys of rainbow trout, Salmo gairdneri, at at the U.C. Davis fish pathology laboratory. Approximately 500 fish exposed to PKX at the American River hatchery were transferred on 9 September 1985 and 15-30 fish samples were taken periodically through April 1985.



Light microscopy. The organism seen at 3 wk was small, condensed, eosinophilic, and contained a daughter cell (Figure 3). Larger, typical parasites were detected in the blood and kidney interstitium the following week (Figure 4). Primary cells with as many as seven daughter cells were evident in these tissues (Figure 5), and were weakly eosinophilic compared to the surrounding host cells. The nuclei of the parasites contained large endosomes that were particularly prominent in the primary cells. The primary cells also contained numerous cytoplasmic granules which stained PAS positive. Degenerating primary cells and small condensed cells were observed in the interstitium of the kidney in some fish. These condensed cells may represent released daughter cells (Figure 6).

Parasites that were in the blood occasionally formed thrombi in the renal vessels (Figure 7), and hypercellularity of the kidney interstitium due to mononuclear cell proliferation and infiltration was associated with PKX infections (Figure 8). Infected fish exhibited a decrease in tubule density when compared with uninfected fish (Figure 9). Many parasites were surrounded by attached macrophages and lymphocytes (Figure 6). Although most fish in this experiment exhibited gross kidney hypertrophy, they did not exhibit other clinical signs of PKD and no significant mortality could be attributed to the parasite.

Organisms with typical PKX morphology were observed in the epithelium of the renal tubules (Figure 10), and the daughter cell of one was seen undergoing binary fission (Figure 11). Apparently some were

degenerating and released their daughter cells into the lumen of the tubule (Figure 12). Small, condensed eosinophilic cells, presumably released daughter cells, were observed in the lumens of tubules at this time. The simplest intraluminal forms were uninucleate cells that resembled the secondary cells of interstitial PKX (Figure 13). More complex intraluminal forms contained secondary and tertiary daughter cells, and continued development of these stages by endogeny and binary fission resulted in sporoblasts with up to six internal sporogonic cells (Figures 14, 15).

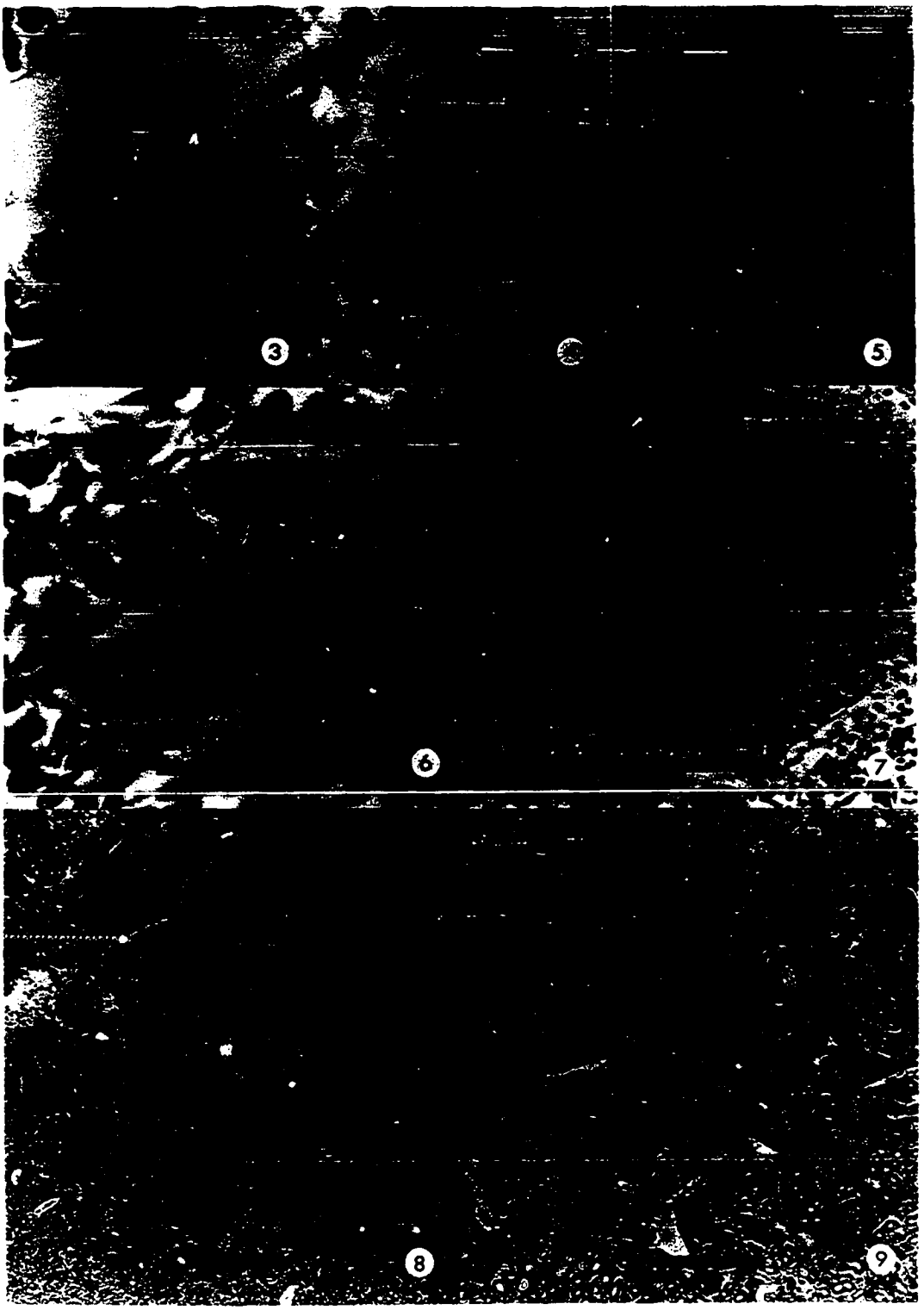
Developing myxosporean spores as evidenced by polar capsule formation were observed in the more advanced intraluminal parasites (Figures 16-18). These spores were oblong, 12 x 7 µm, with two spherical polar capsules 2 µm in diameter (measured from wet mounts, n=20). Capsulogenic nuclei and a large sporoplasm nucleus were prominent in paraffin sections stained with hematoxylin and eosin (Figure 16). The intraluminal organisms were considered to be myxosporean trophozoites because they formed multicellular spores with polar capsules. Only one spore developed within each enveloping cell and no large multinucleated plasmodia, typical of most myxosporeans, were observed. However, up to three nuclei were observed in some enveloping cells. This monosporous development within an enveloping cell, described as a "pseudoplasmodium" by Lom et al. (1982) in Sphaerospora spp., was confirmed in wet mounts (Figure 19). Enveloping cells observed in wet mounts were characterized

by numerous refractile granules, presumably analagous to the PAS positive granules seen in paraffin sections.

No spore valves or clearly defined valvogenic cells were observed in either paraffin sections or wet mounts. The spores were fragile and lacked rigidity as evidenced by their readily changing shape under pressure of the cover slip in wet mounts, and they degenerated within 4 h when minced infected kidneys were maintained in water at 10 C. Furthermore, spores could no longer be detected after 24 h under these same conditions.

The intraluminal trophozoites and spores were most frequently observed in the second proximal and distal segments of the tubules. Although they were not found in the collecting ducts, ureters or urinary bladder, three sporoblasts with polar capsules were observed in the urine examined from one of ten fish.

Figure 3 - 18. Paraffin sections of PKX protozoans and intraluminal sporogonic forms in the kidneys of Salmo gairdneri. P = Primary cells, PN = primary cell nucleus, D = daughter cells, polar pc = polar capsules. M = macrophage. Bar = 10 µm unless otherwise indicated. 3. Earliest recognized PKX in kidney at 3 wk after exposure. H&E. 4. Uninucleate PKX parasites in the interstitium. H&E. 5. Multicellular PKX in renal blood vesssel. H&E. 6. Degenerating PKX primary cells releasing daughter cells. H&E. 7. Thrombus with PKX parasites in renal vessel. H&E. Bar = 50 µm. 8. Interstitial hypercellularity, inflammation and tubular atrophy in the posterior kidney of PKD affected trout. H&E. Bar = 200 µm. 9. Posterior kidney of a normal uninfected trout. H&E. Bar = 200 µm. 10. Epithelium of a renal tubule with PKX. H&E. 11. Secondary daughter cell dividing by binary fission in the epithelium of a tubule. H&E. 12. Primary cell releasing daughter cell into the lumen of a tubule. H&E. 13. Early intraluminal cells. H&E. 14. Interluminal pseudoplasmodia with internal sporoblasts. H&E. 15. Intraluminal sporoblast with cell dividing by binary fission. H&E. 16. Pseudoplasmodium with developing spore. H&E. 17,18. Developing spores in the lumen of a tubule. Giemsa.



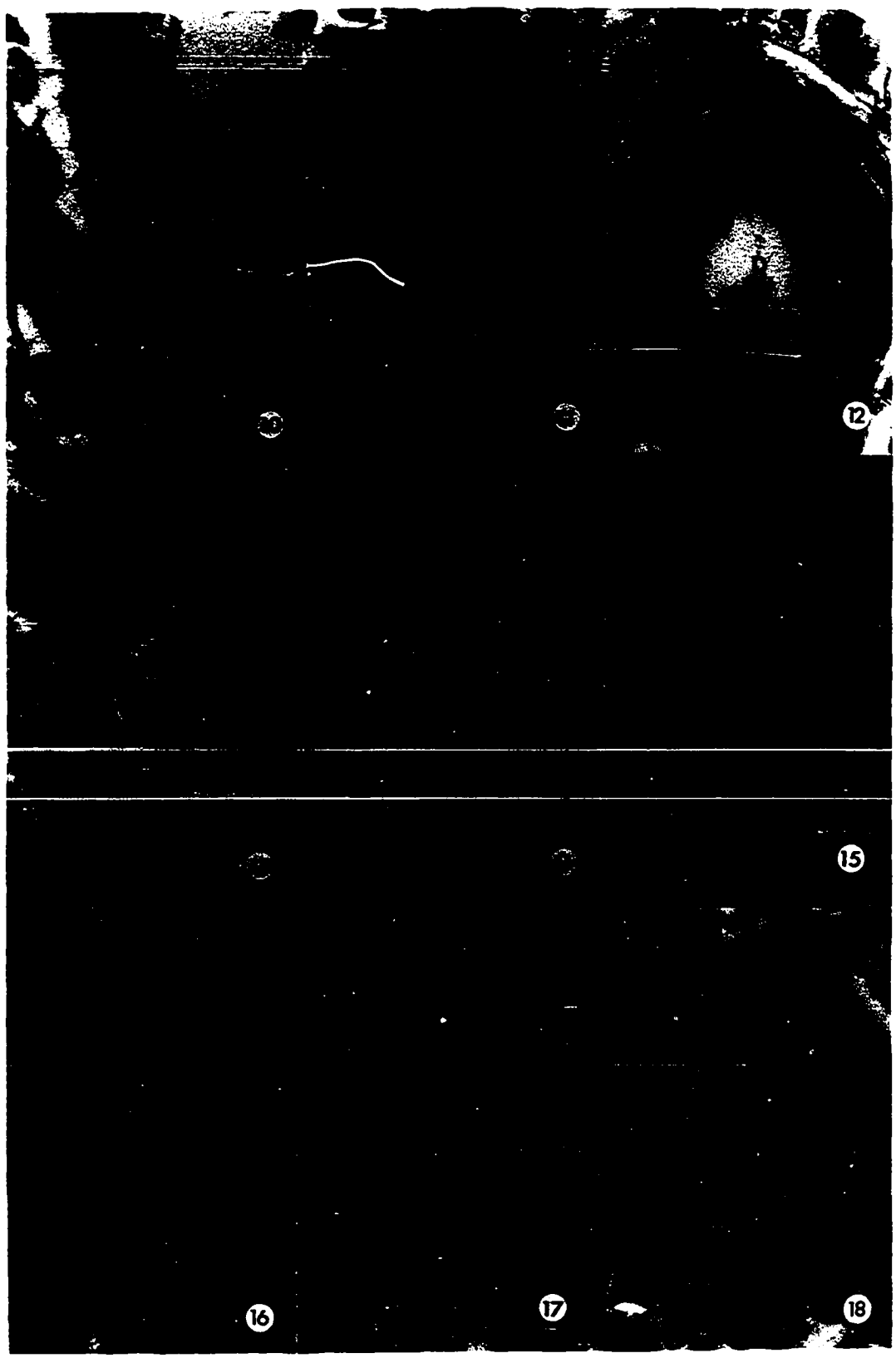
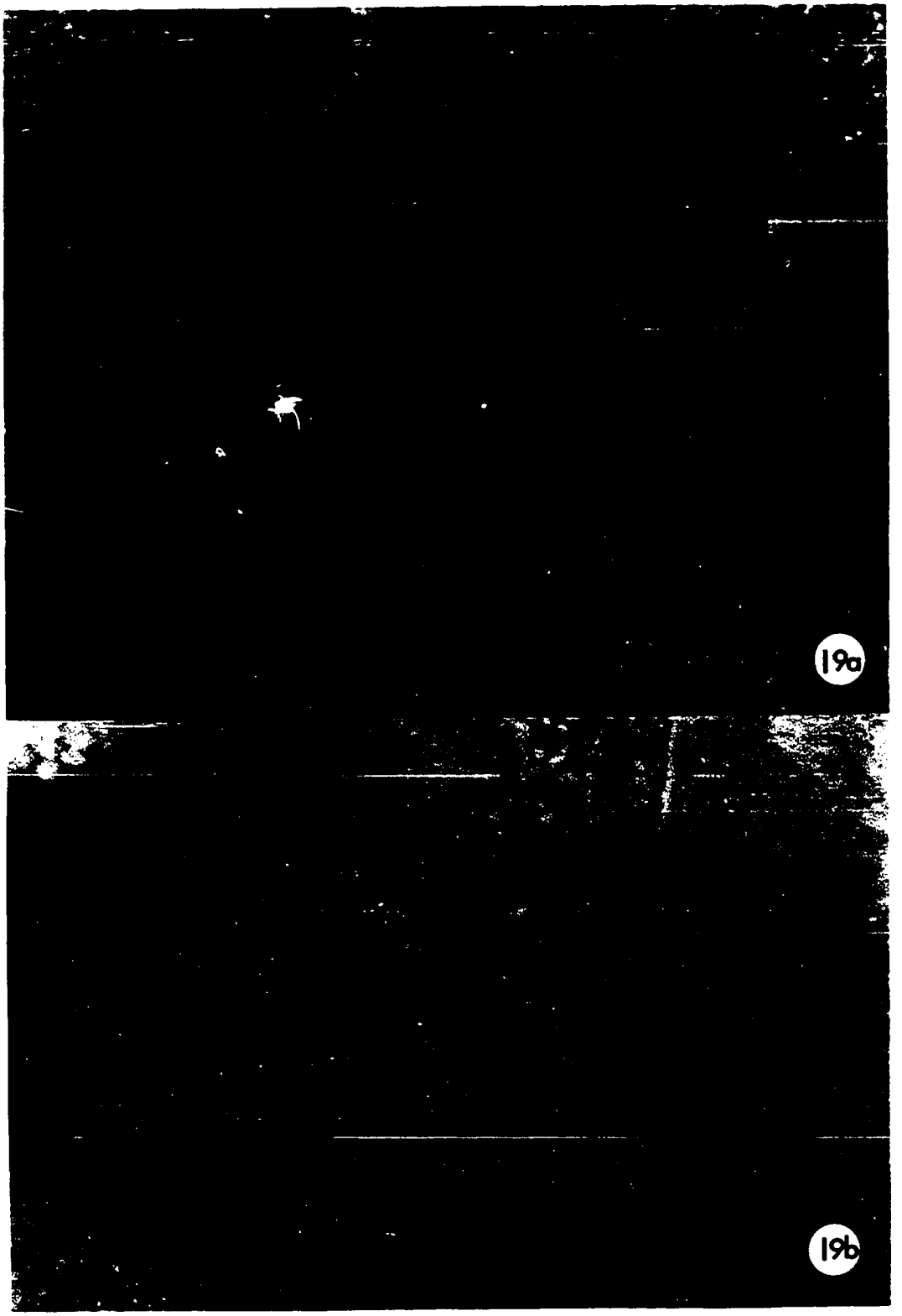


Figure 19. Wet mount of rainbow trout, Salmo gairdneri kidney with intraluminal pseudoplasmodia with developing spores. R = refractile granules, pc = polar capsule. Bar = 10 µm.



Electron Microscopy. The primary cell of PKX often contained internal secondary and tertiary daughter cells (Figure 20) and always "haplosporosomes" identical to those described by Seagrave et al. (1980a,b) (Figure 21). As described by Seagrave et al. (1980a,b), the primary cells contained a prominent endoplasmic reticulum. They also contained large, electron dense membrane bound inclusions which were presumed to be secondary lysosomes. The daughter cells, either secondary or tertiary, were encircled by a membrane of the outer cell. They never contained haplosporosomes and rarely lysosomes. Degenerating primary cells, as evidenced by condensation of organelles and disruption of membranes, with intact daughter cells were observed (Figure 22). The latter may be released to continue the vegetative development of the parasite in the interstitium.

The PKX parasites in the epithelium of the tubules (Figure 23) had an identical ultrastructural morphology to those in the interstitium. They apparently migrated to the lumen by penetrating between the epithelial cells.

The simplest and possibly earliest intraluminal forms were uninucleate cells (Figure 24), and presumably were released daughter cells from typical PKX. These forms and daughter cells in typical PKX shared a similar size, nucleus to cytoplasm ratio, chromatin pattern and plate-like cristae in the mitochondria. Additionally, as with the daughter cells within PKX, they contained little endoplasmic reticula compared to the primary cell of PKX, and had similar ribosome content and cytoplasmic

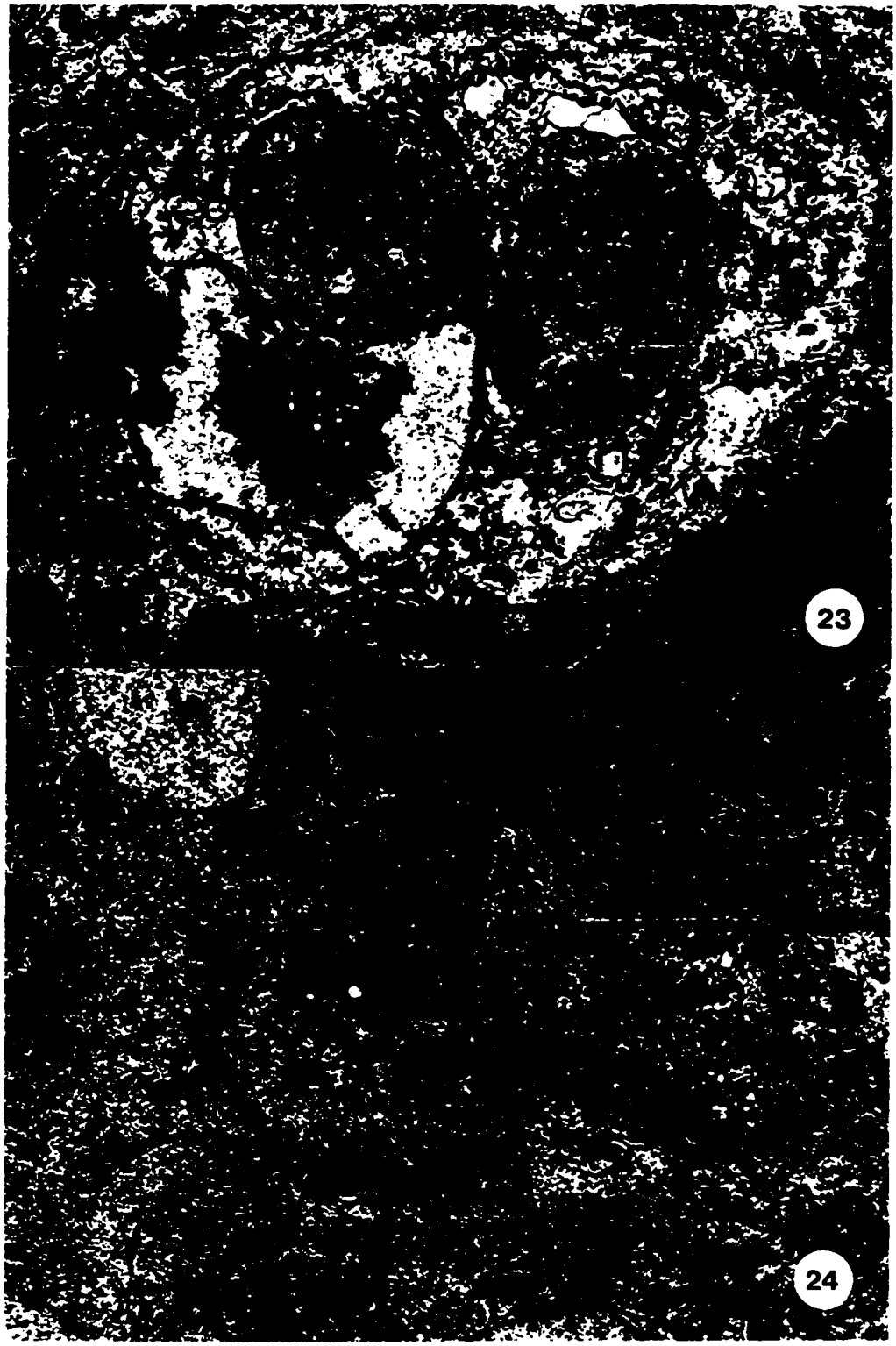
matrices. All of these characteristics resulted in an essentially identical ultrastructural morphology shared by the early intraluminal forms and the daughter cells of typical PKX.

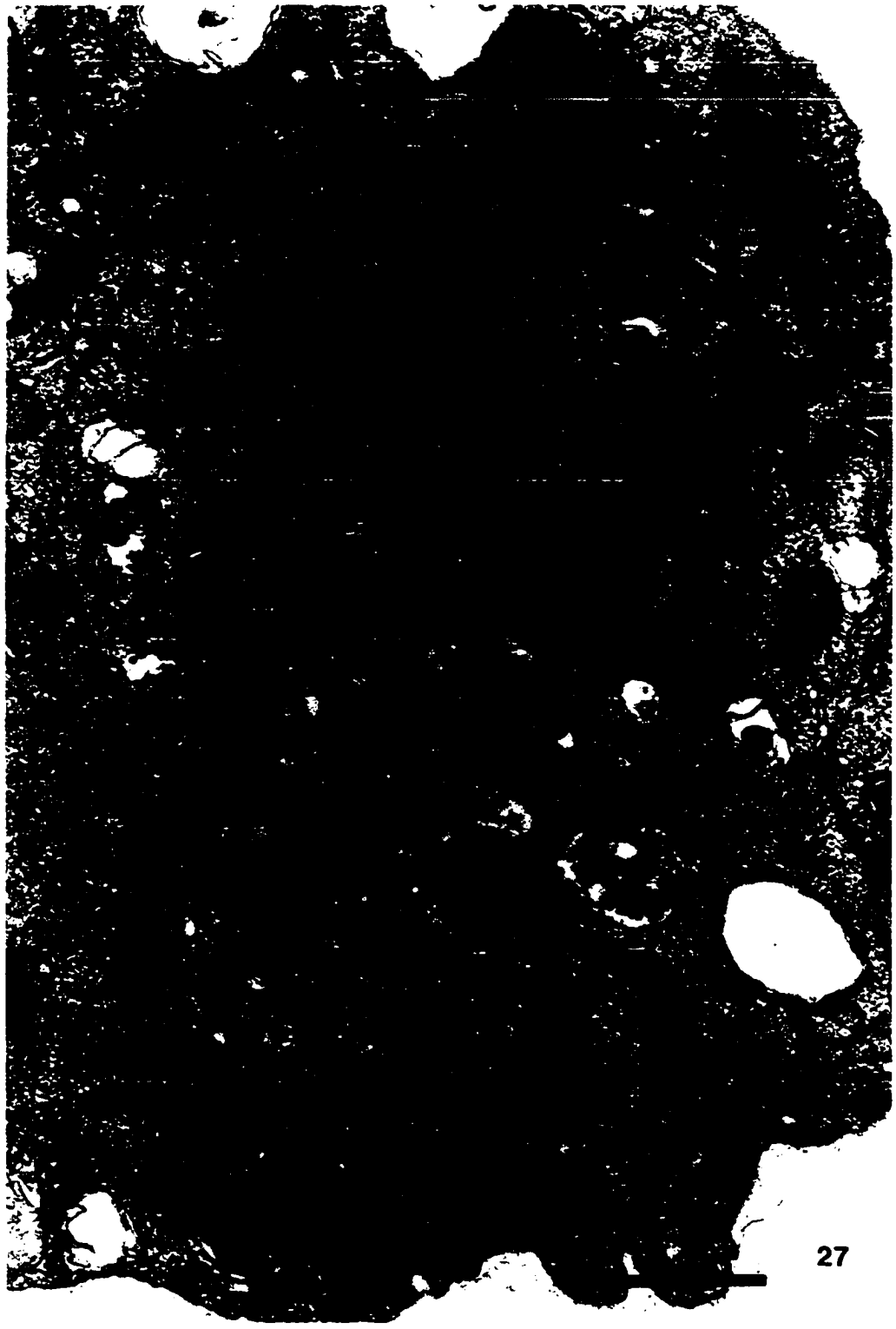
More complex intraluminal forms consisting of an enveloping cell with up to five sporogonic cells were observed (Figures 25, 26). Capsulogenic cells identified by the presence of polar capsule primordia with external filaments were seen (Figure 27), and the most fully developed spores that were observed contained polar capsules with coiled internal filaments (Figure 28). Electron dense multilaminate bodies, most likely corresponding to the PAS positve granules seen by light microscopy, were prominent in the enveloping cells and they apparently condensed as the sporoblast matured (Figures 26-28).

Macrophages usually surrounded the parasites in the blood and kidney interstitium, but they were not observed in association with the intraluminal stages. The only PKX stage that could be considered intracellular were small parasites that had been completely engulfed by a macrophage.

Diagramatic representation of the sequential development of PKX in the kidney of salmonid fishes is shown in Figure 29. All stages represented were observed either by light or electron microscopy.

Figures 20-28. Electron micrographs of typical PKX and intraluminal sporogonic forms in the kidneys of Salmo gairdneri. P = primary cell PN = primary cell nucleus, S = secondary cell, SN = secondary cell nucleus, T = tertiary cell, arrows = haplosporosomes, PC = polar capsule, X = extended polar capsule filament, E = enveloping cell. Bar =2 μm unless otherwise indicated. 20. Interstitial PKX with two secondary cells. Haplosporosomes are confined to the primary cell. 21. Haplosporosomes in a primary cell of interstitial PKX. Bar = 0.2 μm. 22. Degenerating primary cell with intact daughter cells in interstitium. 23. PKX in the epithelium of a tubule. 24. Early intraluminal myxosporean stages in the lumen of a tubule. 25. Pseudoplasmodium with internal sporogonic cells in the lumen of a tubule. 26. Pseudoplasmodium with five sporogonic cells. 27. Capsulogenic cells of an intraluminal sporoblast with a polar capsule primordium and extended polar capsule filaments surrounded by microtubules. 28. a. Intraluminal sporoblast with polar capsules. b. Electron dense multilaminate body seen frequently in enveloping cells. Bar = 0.2 μm. c. Polar capsule with coiled filaments. Bar = 0.2 μm.





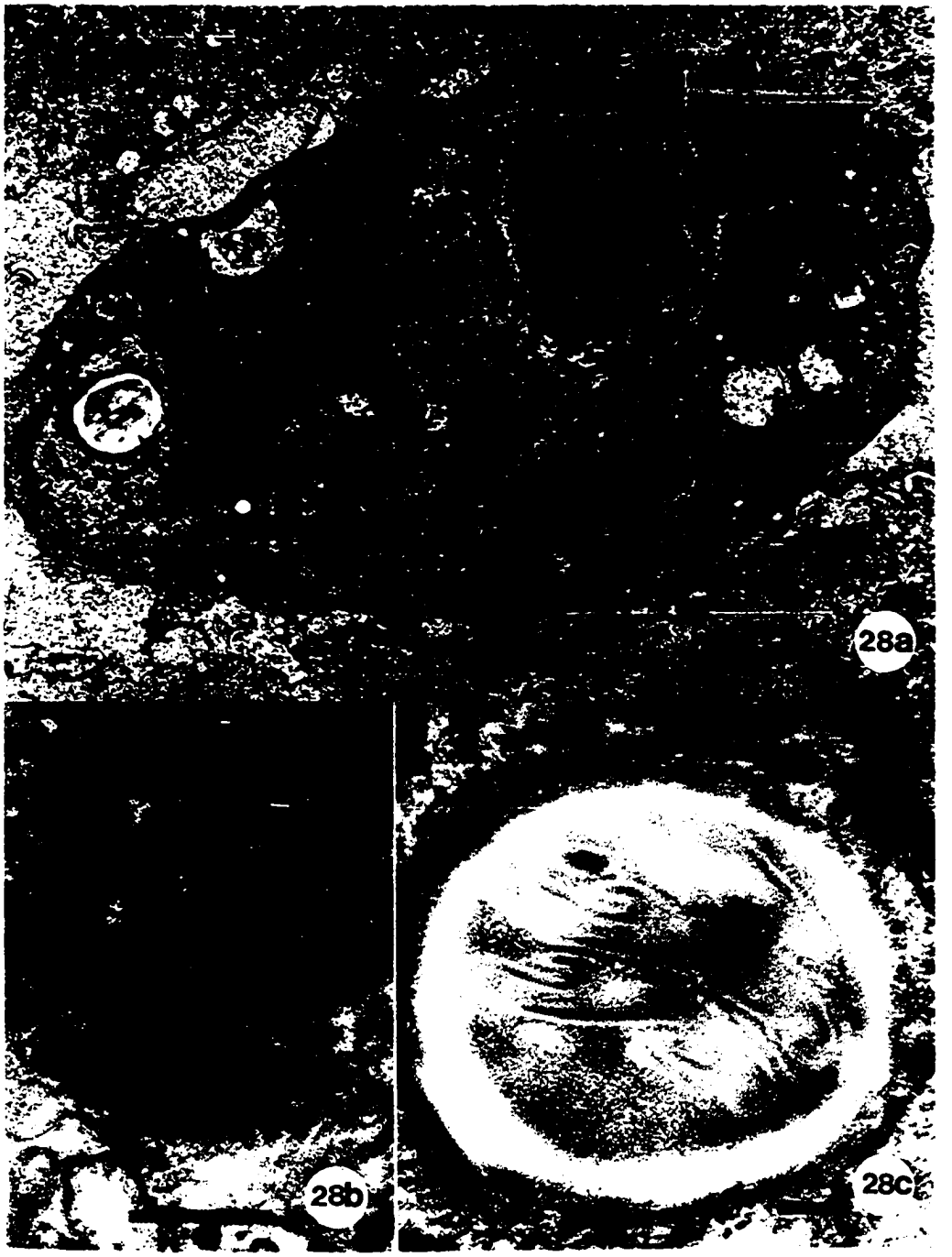
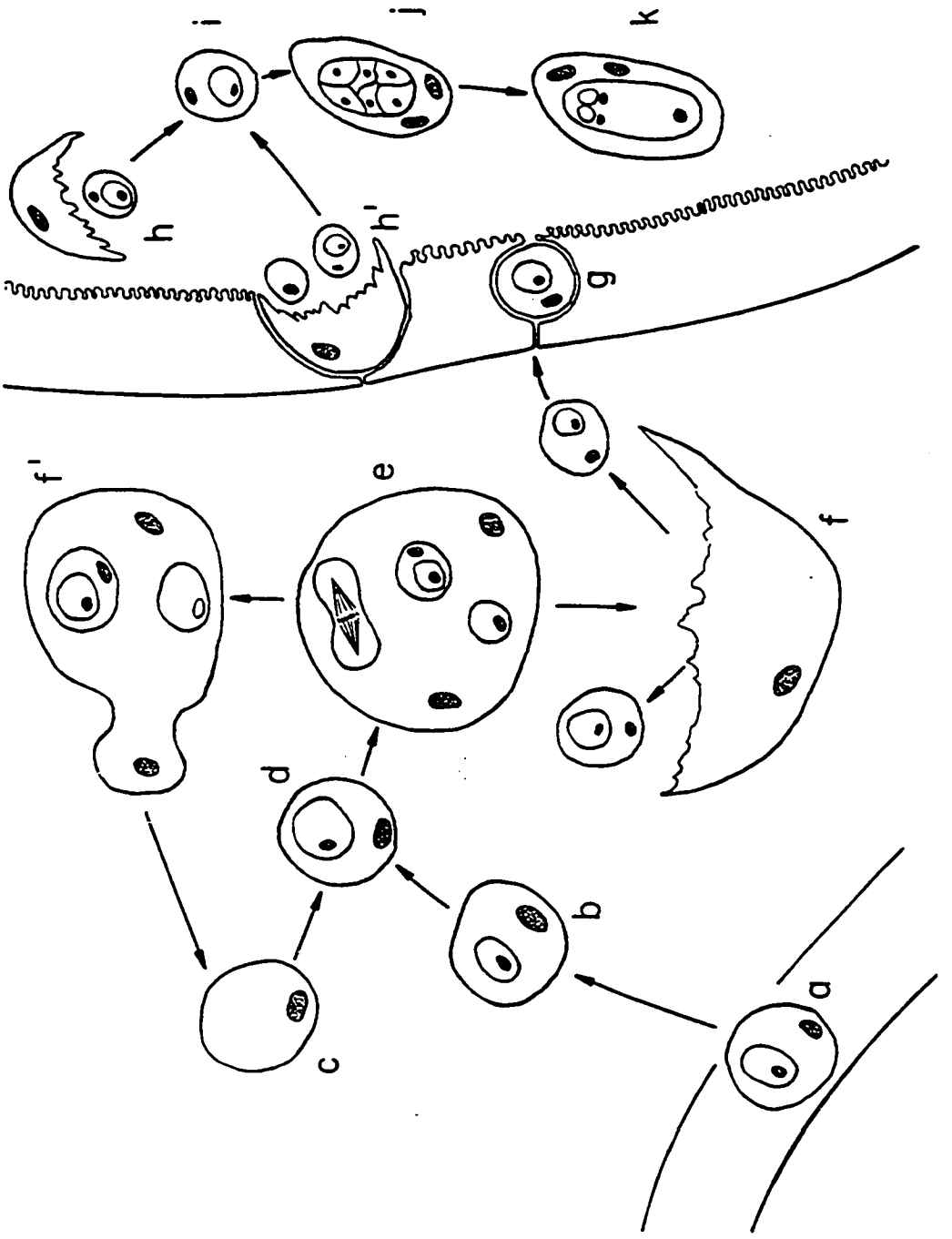


Figure 29. Sequential development of PKX in the kidney of salmonid fishes as interpreted from wet mounts, paraffin sections and electron micrographs. a. The PKX parasite in a blood vessel. b. The PKX enters the kidney interstitium. c. Uninucleate PKX. d. Daughter cell produced by endogenous budding. e. Secondary daughter cells divide by binary fission and produce tertiary cells by endogenous budding. Nuclear division in primary cell may also occur. f. Daughter cells are released from degenerated primary cell and continue vegatative development. f'. Plasmotomy or binary fission of primary cell may also occur. Vegetative development also may occur in the blood vessels. g. PKX parasite penetrates between epithelial cells of the tubule. h. PKX enters lumen; the primary cell degenerates and releases secondary cell (pseudoplasmodium) h'. Primary cells may release daughter cells into lumen from epithelium. Multiplication of released secondary cells may occur in the lumen. i. Released secondary cell (enveloping cell) with tertiary (sporogonic) cell. j. Tertiary cell multiplies resulting in a multicellular sporoblast. k. Sporulation continues; capsulogenic cells differentiate and form polar capsules and sporoplasm develops a prominent nucleus. No valvogenesis observed, and spore remains within the enveloping cell throughout the infection.



Blood and Spleen Transmission of PKX

Parasite-free steelhead trout, S. gairdneri injected with either the blood or spleen tissues of PKX infected rainbow trout became infected with the intraluminal myxosporeans as well as the typical interstitial PKX (Table 1). Control steelhead not exposed to PKX developed neither forms. As observed in the natural exposure experiments, kidney lesions were only observed in fish exhibiting the interstitial stage of PKX. Two of the fish that received blood injections became heavily infected and they showed prominent interstitial nephritis.

Table 1. Prevalence of intraluminal myxosporean and interstitial forms of PKX in the kidneys of steelhead trout, Salmo gairdneri, inoculated into the peritoneal cavity with the blood or spleen from trout with PKD or Minimal Essential Medium (MEM).

	Treatment		
Fish	spleen	blood	MEM
No. with PKX and intraluminal forms	2	3	0
No. with intraluminal forms only	1	3	0
No. with PKX only	0	1	0
Total examined	17	16	20

Effects of cortisol on host response and PKX development.

Rainbow trout were given cortisol implants to determine the effects of immunosuppression on the host response and development of PKX. Indicators of immunosuppression were mortality, leucocrit and plasma cortisol levels, and these were taken at 4 and 8 wk post injection (PI).

Four wk PI. At this time, which was approximately 8 wk following exposure of PKX, the cortisol treated fish had plasma cortisol levels approximately ten times greater than the non-treated groups (Table 2). Furthermore, the treated groups had leucocrits approximately half that of PKX+C- fish (Table 2).

The cortisol treated groups showed mortalities throughout the experiment, while none of the non-treated fish died (Figure 30). The mortalities were due to various opportunistic pathogens. Heavy infections of Gyrodactylus (Monogenea), Costia (Mastigophora), Ichthyophthirius (Ciliata) were seen on the gills and skin, and Aeromonas hydrophila and Pseudomonas sp. were isolated from the kidneys of several moribund or dead fish showing signs of bacterial septicemia.

In fish that received both PKX and cortisol (PKX+C+) the density of interstitial parasites was approximately 20 times greater than the PKX+C- group (Table 2). Although the parasite was detected in 14 of 17 of the PKX+C- fish at 4 wk PI, most were lightly infected. However, the degree of interstitial hypercellularity was greater in this group than all

others as indicated by the lower density of kidney tubules (Table 2). This was due primarily to mononuclear cell proliferation around the parasites (Figure 31). The PKX+C+ fish exhibited less mononuclear proliferation, and the relative increase in interstitium compared to the non-infected groups was due in part to parasites occupying tissue spaces (Figure 32). The intraluminal stages of PKX were detected in all of the PKX+C+ fish, while none were found in the other groups (Table 2).

Cortisol implants in the absence of PKX did not affect the tubule to interstitium ratio. Although some of the PKX-C+ fish had bacterial infections of the kidney, they did not show changes in tubule to interstitium ratio compared to the PKX-C- fish (Table 2).

Eight wk PI. The plasma cortisol levels in the treated groups had diminished at this time, but they still remained considerably higher than the non-treated fish (Table 2). The leucocrits in the PKX+C- fish were no longer different from those of the other groups, and neither interstitial nor intraluminal forms of PKX were detected in this group. The ratio of interstitial tissue to tubules had decreased, and no significant difference in tubule to interstitial ratio was observed between the groups. However, the PKX-C- group had a decrease in the density of tubules when compared to this same group at 4 wk, while no significant change occurred in the PKX+C+ and PKX-C+ groups (Table 2). The PKX+C+ fish remained infected with both forms of PKX, but their intensity of interstitial and intraluminal forms had diminished and the prevalence of

these forms had dropped to 40 and 20 % respectively. Vacuolar degeneration of the tubules was observed in many of the fish from both cortisol treated groups (Figure 33). Seven of the 20 PKX+C+ and one of the PKX-C+ fish exhibited focal interstitial granulomas in the interstitium (Figure 34).

Table 2. Effects of cortisol on the associated pathology and sequential development of PKX. Mean ± SD of leucocrits, plasma cortisol, density of renal interstitial PKX, density of tubules with intraluminal PKX, and tubule density in rainbow trout, *Salmo gairdneri*: PKX infected with cortisol (PKX+C+) and without cortisol (PKX+C-) and PKX-free with cortisol (PKX-C+) and without cortisol (PKX-C-), examined at 4 and 8 wk after cortisol treatment.

		Treatments			
Parameters	wk	PKX+C+	PKX+C-	PKX-C+	PKC-C-
No interstitial PKX/mm^2	4	9.2 ± 9.1	0.5 ± 0.5	0	0
	8	0.13± 0.4	0	0	0
Tubules with intraluminal PKX/mm^2	4	1.0 ± 0.8	0	0	0
	8	0.2 ± 0.3	0	0	0
Tubules/mm^2	4	24.8 ± 4.8	20.2 ± 3.8	30.0 ± 6.0	30.0 ± 3.5
	8	27.0 ± 4.0	24.7 ± 5.0	26.8 ± 3.4	26.4 + 3.1
Plasma cortisol (µg/100 ml)	4	19.1 ± 16.0	1.1 ± 1.2	24.8 ± 10.2	2.7 ± 1.2
	8	6.1 ± 4.3	0.7 ± 0.6	5.6 ± 4.5	0.3 ± 0.3
Leuocrit	4	1.2 ± 0.8	2.5 ± 0.6	1.1 ± 0.5	1.4 ± 0.2
	8	1.4 ± 1.3	1.0 ± 0.5	1.7 ± 0.4	1.5 ± 0.4

Figure 30. Cumulative mortalities in cortisol treated rainbow trout, Salmo gairdneri, with PKX (PKX+C+) and without PKX (PKX-C+).

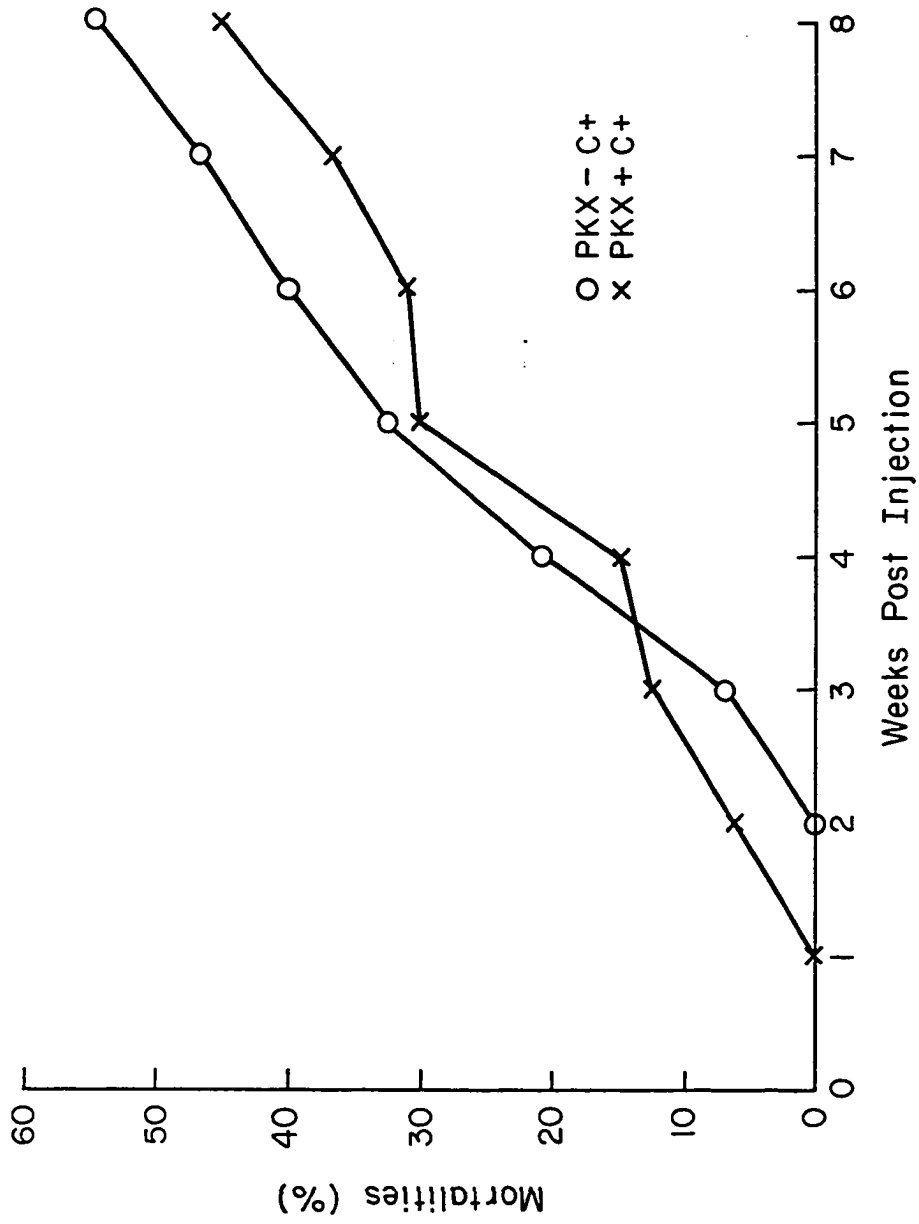


Figure 31. Renal interstitial granulomas surrounding PKX parasites (arrows) in a rainbow trout, Salmo gairdneri, without a cortisol implant. H&E. Bar = 20 µm.

Figure 32. Numerous interstitial PKX parasites (arrows) and intraluminal sporogonic forms (L) in the kidney of a rainbow trout, Salmo gairdneri with a cortisol implant. H&E. Bar = 20 µm.

Figure 33. Vacuolar degeneration of the kidney tubules in a rainbow trout, Salmo gairdneri, with a cortisol implant. H&E. Bar = 30 µm.

Figure 34. Interstitial granulomas (G) in the kidney of PKX-infected rainbow trout, Salmo gairdneri, 8 wk after cortisol implantation. H&E.
Bar = 30 µm.

Discussion

Evidence from this study indicates that PKX belongs to the phylum Myxozoa, and salmonids are most likely abnormal hosts for the parasite. Myxosporeans are ubiquitous, host specific parasites of fishes (Mitchell 1977) and the prespore stages typically do not induce a significant inflammatory response (Dyková and Lom 1978). Salmonids exhibit a severe inflammatory response to interstitial PKX which is the presporogonic form and only immature spores are observed. Although immunosuppression with cortisol implants suppressed interstitial inflammation and enhanced the number of sporogonic forms it did not induce complete sporulation. The blood and spleen transmission experiment verified that typical interstitial PKX and the sporogonic forms in the tubules belonged to the same organism. Epizootiological data and a clear sequential development from early PKX to the sporogonic forms further demonstrated the relationship of the developmental stages of the parasite.

Proliferative kidney disease parasites (PKX) with identical morphology to those reported by Ferguson and Needham (1978), Seagrave et al. (1980a,b) and Smith et al. (1984) were frequently observed in the blood and kidney interstitium. The parasite was detected in fish introduced to water containing the infectious stage one week earlier than previous reports (Ferguson and Ball 1979; D'Silva et al. 1984), and at this time it was smaller and more condensed than typical PKX (Figure 3). Numerous typical forms were observed in the blood, particularly early in

the infection, which indicates that it reaches the kidney via the circulatory system.

The PKX parasite most likely undergoes vegetative reproduction within the fish host. This is supported by the observations of numerous daughter cells in the kidney interstitium of naturally infected fish. Additionally in the blood and spleen transmission study some fish injected with very few parasites became heavily infected, and fish transferred from the hatchery to PKX-free water at U.C. Davis before parasites could be detected, later developed heavy infections (Figure 2). This indicates that continual exposure is not needed to induce heavy infections. A mechanism for the development of the endogenous cells by internal cleavage was reported by Seagrave et al. (1980a,b). However, binary fission of the parasites internal cells, as reported for the first time in the present study, demonstrates a second method for vegetative reproduction. No fixed sequential development within the kidney interstitium could be determined, and plasmotomy (external budding), endogeny (internal budding) and binary fission may all occur in the same parasite (Figure 29). This lack of rigidity in the vegetative development is consistent with the phylum Myxozoa (Lom et al. 1983; Johnstone 1984). However, there may be a fixed number of asexual generations before sporulation takes place, as occurs with coccidians (phylum Apicomplexa). This might explain why transmission of the parasite beyond two passes by intraperitoneal injection was unsuccessful in the present study.

Ferguson and Needham (1978) reported PKX in the renal tubules, but migration of the parasite through the epithelium and release of daughter cells into the lumen was not described. The PKX parasites in the epithelium of the tubules and the intraluminal stages were prevalent in convalescing fish (Figure 1) and as Ferguson and Needham (1978) suggested, the tubules may provide a mode of exit for the parasite.

The simplest intraluminal stage detected in this study was a small unicellular organism. Similar forms contained single endogenous daughter cells (Figure 24). These stages appeared identical to the internal daughter cells of the interstitial and epithelial PKX (Figures 20, 23) and are most likely the same cells. Haplosporosomes are only observed in the primary cell of PKX (Ferguson and Needham 1978; Seagrave et al. 1980a; Smith et al. 1984). This accounts for their absence in the intraluminal forms because this stage seems to arise from released secondary cells of typical PKX. The intraluminal forms represent pseudoplasmodia and the internal cells are the sporoblast, as described by Lom et al. 1982 for Sphaerospora. The enveloping cell of the pseudoplasmodium of Sphaerospora is uninucleate (Lom et al. 1982), but the enveloping cell of PKX often contained two or three nuclei. Polar capsule formation in PKX indicated spore formation. However, PKX trophozoites were more numerous than spores, even late in the infection, as was reported by Bond (1938) for Sphaerospora renicola in the killifish, Fundulus heteroclitus L.

Polar capsules may form before valvogenesis occurs as in other myxosporeans (Desser and Paterson 1978; Current et al. 1979), and valves

were not observed in the PKX spore. The spores remained within their pseudoplasmodia throughout the infection. This, combined with the lack of valves indicates that the spores never fully developed. Five to six sporogonic cells were observed in maturing sporoblasts, which is consistent with myxosporean sporogenesis. Two of the cells correspond to the capsulogenic cells, one or two to the sporoplasm cells, and presumably two for the valve forming cells (Mitchell 1977). Although valves were not observed, PKX spores most likely form vestigial valvogenic cells in salmonids. These spores were fragile, and it is unlikely that they would survive long enough to form valves after they are released from the fish.

A clear sequential development could be followed from the interstitial PKX to these spores by light and electron microscopy, which demonstrated the myxosporean nature of the parasite. This is further supported by epizootiological evidence and experimental data. The myxosporeans of this study were only observed in PKX exposed fishes, and although they have not been reported previously, they have been detected in kidney sections of PKD infected fish from all known areas where the disease has occurred. These include two enzootic areas on Vancouver Island, B.C., Canada, in convalescing rainbow trout from the Hagerman State hatchery, Idaho, USA and from all locations in California. Similar stages have been observed in Atlantic salmon and brown trout in Europe (R. S. Clifton-Hadley, Ministry of Agriculture, Fisheries and Food, Weymouth, England, pers. comm.). The coincidence of typical PKX with the later sporulating forms was further shown in studies by Hedrick et al. (1985).

Fish introduced to the American River hatchery during the spring and summer showed both typical PKX and the intraluminal myxosporeans, whereas fish exposed in the winter months when the infectious stage was not present did not become infected with either stage of the parasite. The blood and spleen transmission experiment further substantiates that both forms belong to the same parasite. Fish injected with the blood and spleen of PKX infected fish developed both interstitial PKX and the intraluminal myxosporeans. Since only typical PKX is found in the blood and spleen, the myxosporeans must have arisen from this stage.

Although Seagrave et al. (1980a) suggested that PKX is a haplosporidan (phylum Acetospora) because of its similarities with the invertebrate pathogen Marteilia, they did not exclude the possibility that it may belong to the phylum Myxozoa. The higher taxonomy of these phyla is in confusion. Desportes and Ginsburger-Vogel (1977) demonstrated affinities of the family Marteiliidae with the classes Paramyxea (Acetospora), Actinosporea and Myxosporea (Myxozoa), and they suggested they be grouped in a common taxon. Most of the myxosporeans examined by transmission electron microscopy form electron dense cytoplasmic inclusions in the sporoplasm similar to haplosporosomes in the Acetospora (Current and Janovy 1977; Desser and Paterson 1978; Current et al. 1979; Lom et al. 1982; Desser et al. 1983), and members of both phyla reproduce by internal cleavage.

Although the complete taxonomy of the PKX myxosporean could not be determined because only immature spores were found, it has similarities to

that of at least three other myxosporean genera: Sphaerospora, Mitraspora and Parvicapsula. Many similarities exist between the prespore form of PKX and a protozoan of carp blood, which was described as a possible early myxosporean stage by Bucsek and Csaba (1981). Lom et al. (1983) reported that this parasite, designated UBO or Csaba's blood protozoan, may be an early stage of Sphaerospora renicola. A protozoan similar to UBO found in the swimbladder of carp was shown to be an early stage of S. angulata (Molnár 1984). The PKX parasite is similar to UBO and the swimbladder protozoan in that all three form endogenous secondary and tertiary daughter cells which are released and may subsequently sporulate in the lumens of the kidney tubules. As with PKX, the secondary cells of UBO reproduce by binary fission in addition to endogenous budding. Furthermore, sporogenesis of PKX is similar to that of Sphaerospora spp. in that spores of both are formed within a "pseudoplasmodium" in the lumens of the kidney tubules, and PKX is monosporous like S. molnari (Lom et al. 1983).

A comparison of immature spores of Sphaerospora to those of PKX show similar features such as an elongated shape, small polar capsules and indistinct valves. In contrast to PKX spores, the mature spores of Sphaerospora have a spherical shape and prominent valves. Ferguson (1984) reported Sphaerospora in the kidneys of feral brown trout from PKD enzootic waters, and Lom et al. (1983) observed pseudoplasmodia without spores in brown trout and grayling in Czechoslovakia. These fishes are

hosts for PKX, and the pseudoplasmodia were possibly the intraluminal stages of PKX.

The PKX parasite also shows some affinities with Mitraspora, which is another genus in the family Sphaerosporidae. Mitraspora cyprini Kudo causes severe kidney hypertrophy in goldfish, Carassius auratus (L.), and spores are rarely found because fish often die prior to sporulation (Hoffman 1984). The spores also resemble those of PKX in that they are elongated. However, PKX trophozoites do not develop into large pansporoblasts as with Mitraspora (Ahmed 1973). Additionally, Mitraspora proliferates within the epithelial cells of the tubules which results in papillary cystic hyperplasia (Hoffman 1984), instead of interstitial hyperplasia which is characteristic of PKD.

Although the PKX myxosporean shows some similarities to members of the family Sphaerosporidae, its spore also resembles those of Parvicapsula (family Parvicapsulidae). Members of this genus are also elongated and have two polar capsules similar in size to those of PKX. Furthermore, a Parvicapsula sp. is known to sporulate in the tubule epithelium in the kidney of coho salmon (Hoffman 1984; Johnstone 1984). Although Parvicapsula forms pansporoblasts, it may also form pseudoplasmodia (J. Lom, Czechoslovak Academy of Sciences, Č. Budějovice, Branišovská, Czechoslovakia, pers. comm.; Johnstone 1984).

Spore morphology has been the most important criterion for species descriptions in the Myxozoa (Mitchell 1977), but the origin of sporobloasts (either within true plasmodia or pseudoplasmodia) should also

be included in these descriptions (Lom et al. 1982; Lom and Noble 1984). Identification of the primary host and completely formed spores, however, are needed before the precise taxonomic status of the PKX myxosporean can be determined.

Severe inflammation is associated with the presporogonic forms of PKX found in the kidney interstitium, and this is in contrast to the lack of tissue reaction to this stage in hosts in which myxosporeans complete sporogenesis (Dyková and Lom 1978). No significant pathology was associated with the intraluminal stages of PKX, and the interstitial nephritis disappeared shortly after the typical PKX form was no longer detected (Figure 1). Ferguson (1981) reported that fish with PKD recover faster in cold water. However, low temperatures are not required for the disappearance of the interstitial PKX, recovery from PKD, migration of PKX to the tubules and sporulation, because all of these occurred in the fish transferred to the U.C. Davis laboratory where temperatures remained above 15 C (Figure 2). Furthermore, spores were detected in another group of rainbow trout which were maintained at a constant 21 C.

This suggests that the stage of development of the parasite is an important factor in the inflammation associated with PKD. Correspondingly, the inflammatory response in an abnormal host may inhibit the migration of PKX to the tubules and subsequent sporulation. Cortisol implants have been used to modify the inflammatory response in fishes (Robertson et al. 1963; Hill and Fromm 1968; Pickering and Duston 1983), and high plasma cortisol levels, depressed leucocrits and mortalities due

to opportunistic pathogens are all associated with immunosuppression in fish. Therefore, these parameters indicated that the fish in the present study were immunosuppressed. Pickering and Duston (1983) and Robertson et al. (1963) reported chronic mortalities to be characteristic in fish immunosuppressed with cortisol implants, and approximately 50% of the cortisol treated fish in the present study died from infections of Gyrodactylus, Costia, Ichthyophthirius, Aeromonas hydrophila and Pseudomonas sp. (Figure 30). These are ubiquitous opportunistic pathogens of cultured fishes and usually cause disease only in stressed fish (Wedemeyer and Wood 1974). No mortality occurred in the groups without cortisol implants (PKX+C-, PKX-C-), and mortality rates of the two cortisol treated groups were similar to each other. Therefore, no mortality could be attributed to PKX.

The plasma cortisol levels in the implanted fish were approximately 10 times greater than the controls at 4 wk post injection (PI) (Table 2) and were greater than those reported by Pickering and Duston (1983), in which brown trout with cortisol implants succumbed to Aeromonas salmonicida infections. Although the levels in the present study were lower than those of Robertson et al. (1963) and Hill and Fromm (1968), they were within the range of those achieved by stressing fish with transport or confinement (Strange and Schreck 1978; Strange et al. 1978).

Robertson et al. (1963) and Pickering and Duston (1983) reported peak blood cortisol levels 2-3 wk after implantation, and the plasma cortisol levels in the present study were higher at 4 wk PI than 8 wk. However,

the plasma cortisol levels of the treated fish at 8 wk were still considerably higher than the controls. Mortalities in the cortisol treated fish occurred at a constant rate over the 8 wk period (Figure 30). This, with the high plasma cortisol levels at both 4 and 8 wk, indicated that these fish were immunosuppressed throughout the study.

Anderson et al. (1982) and McLeay (1973) reported lower lymphocyte counts in fish immunosuppressed with corticosteroids, but the cortisol treated fish in the present study did not have significantly lower leucocrits than the PKX-C- fish. However, at 4 wk PI, the PKX+C- fish had leucocrits twice that of the PKX+C+ group. This indicates that leucocytosis occurred in the PKX+C- fish but was suppressed in the PKX+C+ fish.

Kidney tubule degeneration in cortisol treated fish was reported by Robertson et al. (1963) and was observed in some of the fish from both cortisol treated groups in the present study. However, Aeromonas hydrophila and Pseudomonas infections occurred in many of the fish with cortisol implants, and it is not known if the kidney lesions were due directly to the cortisol or to the bacteria.

The suppressed inflammatory response clearly enhanced the development of PKX and was reflected in the histological sections. Although the PKX+C+ fish had approximately 20 times the density of interstitial PKX parasites, they exhibited less interstitial hypercellularity (Table 2). Intraluminal forms of PKX were detected in all of the PKX+C+ fish at 4 wk PI (which was 8 wk after exposure to PKX), while none were ever detected in the PKX+C- fish. Possibly suppression of the inflammatory response in the former group allowed greater vegetative development of the parasite in the blood and interstitium. Additionally, more parasites reached the sporogonic stage, which resulted in an abundance of intraluminal forms in the cortisol treated group (Table 2).

By 8 wk PI (12 wk after exposure to PKX), the PKX+C- fish had recovered from PKD. Typical interstitial PKX parasites were absent, the interstitial inflammation had subsided and leucocrits had dropped to the level of the other groups (Table 2). As with the 4 wk sample, no intraluminal forms were detected in the PKX+C+ fish. In contrast, fish maintained at the hatchery and the laboratory the previous year exhibited the intraluminal forms (Figure 1, 2), but these fish had considerably higher intensities of infection with interstitial PKX. Because the PKX+C- fish were lightly infected and immunocompetent, their inflammatory response to the parasite in the interstitial phase was probably effective in eradicating the infection before the intraluminal forms could be produced. This did not occur in the PKX+C+ fish and they were still infected with interstitial and intraluminal sporogonic forms of PKX at 8

wk PI. Some of these fish also exhibited focal interstitial granulomas (Figure 34), but it was not determined if these were due to PKX or chronic bacterial infections because these lesions were also observed in one PKX-C+ fish.

This immunosuppression experiment has shown that the intensity of PKX and associated inflammation depends on the immune status of the fish, and may help to explain differences in pathology seen in fish affected by PKD. Stressful stimuli (i.e. crowding, starvation and poor water quality) may induce high blood cortisol levels (Donaldson 1981), and result in immunosuppression. Therefore, these factors may account for the severe interstitial inflammation seen in some fish with PKD, while others (presumably less immunocompetent) may be infected with numerous PKX organisms and show little tissue reaction. Additionally, studies by Manning et al.(1982) have shown that older fish are usually more immunocompetent, and Hedrick et al. (1985) reported greater tissue reaction and fewer intraluminal forms of PKX in older fish with PKD than younger fish maintained at the same hatchery.

Spores and numerous sporoblasts were observed in the PKX+C+ fish, but none were further developed than those seen in natural epizootics. Therefore, although suppression of the inflammatory response allowed more parasites to reach the sporogonic stage, it did not induce complete development of the spore in the rainbow trout. Possibly the kidney tubules of this fish do not provide the proper physiological environment for complete sporulation. This is substantiated by the observations from

fish in natural epizootics, in which spores persisted in an immature state in the tubules several months after the inflammation had subsided (Figure 1).

This study has demonstrated that the causative agent of PKD is the presporogonic form of a myxosporean, which undergoes vegetative development and induces a severe tissue response in the kidney interstitium and blood of rainbow trout. As the disease progresses, the parasites migrate to the lumens of the kidney tubules and sporulate within psuedoplasmodia. Although inflammation was not associated with these sporogonic forms, only immature spores were observed, even in immunosuppressed fish. These all indicate that salmonids are abnormal hosts for PKX, and the parasite may complete sporulation in a non-salmonid fish.

Summary and Conclusions

1. The development of the PKX myxosporean, the causative agent of proliferative kidney disease of salmonid fishes, was described by light and electron microscopy.

2. PKX undergoes vegetative development by binary fission and endogeny in the kidney interstitium where it invokes an interstitial nephritis.

3. As the disease progresses, parasites migrate to the tubules and sporulate. Monosporous spores are formed within pseudoplasmodia similar to Sphaerospora spp., but complete sporogensis was not observed.

4. Description of the sequential development from typical interstitial PKX to to the intraluminal myxosporeans, epizootiological data and the blood and spleen transmission experiment verified that the interstitial and intraluminal forms belonged to the same organism.

5. Therefore, PKX is a member of the phylum Myxozoa.

6. Fish given cortisol implants were immunosuppressed as indicated by high plasma cortisol levels, depressed leucocrits and chronic mortality due to opportunistic pathogens.

7. The immunosuppressed fish that were infected with PKX exhibited twenty times the density of interstitial PKX but less interstitial hypercelluarity than immunocompetent fish with PKX.

8. All of the cortisol treated fish with PKX showed intraluminal sporogonic forms at 4 wk post injection, and none were detected in the non-treated fish.

9. Suppression of the inflammatory response allowed more parasites to reach the sporogonic stage, but it did not induce complete sporulation.

10. Salmonids are most likely abnormal hosts for PKX, and it may complete sporulation in a non-salmonid fish.

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Appendix

Leucocrits, plasma cortisol, tubule and PKX density in: PKX infected with cortisol (PKX+C+); PKX infected, not cortisol treated (PKX+C-); PKX-free, treated with cortisol (PKX-C+) and control (PKX-C-) rainbow trout, Salmo gairdneri, at 4 and 8 wk post cortisol implantation. * = significant at $p<0.01$.

Leucocrit (%) 4 wk

Fish #	PKX+C+	PKX+C-	PKX-C+	PKX-C-
1	1.21	2.30	1.40	1.26
2	1.50	3.00	0.60	1.19
3	1.74	2.18	0.80	1.16
4	1.63	3.15	0.70	1.84
5	1.84	2.03	1.12	1.16
6	1.88	1.62	0.90	1.40
7	0.14	1.90	1.35	1.64
8	0.36	2.06	0.13	1.26
9	1.09	4.13	1.43	1.80
10	1.89	2.70	0.44	1.06
11	0.48	1.54	0.99	1.83
12	0.16	2.80	1.47	1.35
13	3.08	2.80	0.76	1.90
14	0.25	2.56	0.87	1.65
15	0.50	3.15	1.67	1.80
16	1.06	2.52	1.93	1.40
17	1.36	2.86	1.31	0.95
X	1.19	2.54	1.05	1.46
SD	0.80	0.64	0.22	0.19

F = 61.70*
LSD = 0.51

Leucocrit (%) 8 wk

Fish #	PKX+C+	PKX+C-	PKX-C+	PKX-C-
1	6.50	1.44	1.12	1.45
2	0.47	1.60	1.17	1.59
3	1.05	2.02	0.74	2.19
4	1.39	1.60	1.54	2.80
5	0.87	0.95	0.55	1.26
6	1.53	1.30	0.35	1.07
7	1.28	1.46	0.51	2.01
8	1.43	1.02	1.20	1.15
9	0.38	1.33	1.93	2.10
10	1.12	1.59	0.72	1.15
11	1.15	1.64	1.07	1.78
12	1.34	1.78	0.92	0.97
13	1.08	2.17	1.00	1.00
14	1.01	1.67	1.63	1.17
15	2.50	1.97	0.42	1.54
16	0.56	2.03		1.28
17	1.40	2.00		1.72
18	1.26	2.29		1.12
19	0.62	1.99		1.52
20	0.50	2.03		1.74
X	1.37	1.73	0.99	1.54
SD	1.29	0.40	0.46	0.54
F = 2.75				
t (4 vs 8 wk)	0.57	50.3*	0.34	1.01

Plasma cortisol (µg/100ml) 4 wk

Fish #	PKX+C+	PKX+C-	PKX-C+	PKX-C-
1	4.9	0.14	29.6	2.1
2	8.5	2.40	18.4	4.8
3	18.8	3.00	29.2	2.2
4	17.5	2.00	42.3	3.5
5	5.4	1.80	23.8	4.2
6	7.6	4.30	40.4	2.2
7	27.9	0.25	12.8	4.7
8	23.8	0.59	19.8	4.7
9	22.8	0.82	22.3	2.7
10	6.2	0.15	15.3	2.0
11	18.9	0.66	27.6	2.7
12	39.7	0.10	24.0	1.8
13	8.3	0.08	22.8	1.2
14	69.2	0.25	26.9	2.0
15	24.0	0.30	17.8	2.6
16	15.5	0.68	15.9	2.9
17	5.7	0.85	41.5	2.7
X	19.1	1.0	24.8	2.7
SD	16.0	1.2	10.2	1.2

F = 25.6*
LSD = 8.5

Plasma cortisol 8 wk

Fish #	PKX+C+	PKX+C-	PKX-C+	PKX-C-
1	4.7	0.03	3.9	0.24
2	9.3	1.6	3.6	0.0
3	5.5	0.58	9.3	0.20
4	1.4	0.14	2.0	0.14
5	7.3	0.64	3.2	0.16
6	5.2	1.9	15.9	0.61
7	3.4	0.69	3.8	0.45
8	1.8	0.57	4.6	0.45
9	5.7	1.1	1.2	0.64
10	4.8	2.3	3.0	0.40
11	4.0	0.32	12.1	0.31
12	1.6	0.22	8.4	0.33
13	8.5	0.42	3.3	0.36
14	15.0	0.45	0.12	1.1
15	5.0	0.58	10.1	0.17
16	3.5	0.81		0.20
17	6.0	0.54		0.06
18	3.0	0.69		0.23
19	18.6	0.52		0.76
20	7.2	0.41		0.14
X	6.1	0.73	5.6	0.35
SD	4.3	0.58	4.5	0.27
F = 18.6*				
LSD = 2.6				
t (4 vs 8 wk)	3.5*	0.92	6.6*	8.5*

Number of interstitial PKX/mm^2
(No parasites detected in PKX+C- at 8 wk)

	4 wk		8 wk
	PKX+C+	PKX+C-	PKX+C+
1	27.0	1.56	0.00
2	34.0	0.27	0.00
3	4.0	0.34	0.00
4	11.1	0.82	0.00
5	4.2	0.20	0.00
6	0.5	0.54	0.19
7	0.3	1.02	0.00
8	10.3	0.07	0.00
9	19.5	0.00	0.54
10	19.3	2.04	0.00
11	7.8	1.29	0.07
12	8.8	0.20	0.00
13	15.4	0.00	0.07
14	16.8	0.00	0.00
15	4.2	0.68	0.00
16	10.6	0.48	0.00
17	7.3	0.75	0.00
X	9.2	0.49	0.13
SD	9.1	0.50	0.40

t (PKX+C+ vs PKX+C-) = 4.8* at 4 wk, 1.29 at 8 wk

t (PKX+C+ 4 vs 8 Wk) = 10.5*

t (PKX+C- 4 vs 8 wk) = 4.4

Number tubules/mm^2 with intraluminal PKX in PKX+C+ fish
(no intraluminals detected in PKX+C- fish)

	4 wk	8 wk
1	0.48	0.07
2	0.68	0.61
3	0.20	0.00
4	2.72	0.00
5	1.09	0.82
6	0.07	0.88
7	0.20	0.07
8	0.68	0.00
9	2.72	0.14
10	0.14	0.48
11	1.25	0.68
12	2.11	0.00
13	1.15	0.14
14	0.74	0.00
15	1.09	0.00
16	0.34	0.00
17	1.84	0.00
X	1.03	0.19
SD	0.85	0.31

t (4 vs 8 wk) = 10.5*

Number of tubules/mm^2 at 4 wk

Fish #	PKX+C+	PKX+C-	PKX-C+	PKX-C-
1	26.4	19.1	35.1	34.0
2	27.3	21.9	35.2	29.7
3	24.5	13.9	31.1	30.5
4	28.4	26.4	29.7	28.7
5	16.5	20.6	31.0	35.5
6	15.6	18.2	35.2	28.9
7	17.8	18.0	24.4	32.7
8	22.2	16.9	29.7	32.0
9	24.2	18.2	37.2	30.7
10	23.0	21.8	26.9	33.8
11	32.9	19.1	21.9	26.2
12	29.1	18.3	30.2	23.8
13	28.3	26.4	24.0	32.7
14	27.9	27.5	32.8	33.1
15	25.8	21.9	33.2	23.1
16	26.6	23.5	39.6	30.9
17	30.6	16.1	18.8	27.6
X	24.8	20.2	30.0	30.0
SD	4.8	3.8	6.0	3.5

F = 19.9*
LSD = 3.89

Number of tubules/mm^2 at 8 wk

Fish #	PKX+C+	PKX+C-	PKX-C+	PKX-C-
1	24.3	23.4	28.3	29.2
2	22.0	28.0	22.4	31.0
3	34.1	21.3	30.8	29.0
4	34.8	30.2	25.3	21.0
5	33.3	25.4	21.8	24.7
6	26.8	32.3	25.2	25.5
7	30.0	32.6	29.0	23.9
8	23.7	36.4	23.0	22.1
9	26.3	26.2	27.6	21.9
10	27.2	19.1	33.0	24.9
11	32.8	23.9	26.9	25.7
12	24.0	22.3	28.5	29.1
13	26.3	27.5	30.4	30.2
14	27.4	18.0	22.3	24.4
15	18.2	23.4	26.7	26.8
16	25.5	24.5		25.1
17	30.4	19.5		27.7
18	25.9	21.5		22.3
19	31.0	24.4		30.8
20	26.4	21.8		23.1
X	27.0	24.7	26.8	26.0
SD	4.0	5.0	3.4	3.1
F	1.3			
t (4 vs 8 wk)	1.5	3.1*	0.6	3.7*