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Effect of Proliferative Kidney Disease (PKD) on Blood and Immunological Parameters in Rainbow Trout (Oncorhynchus mykiss).

BY

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Bachelor of Science, 1982
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DISSERTATION

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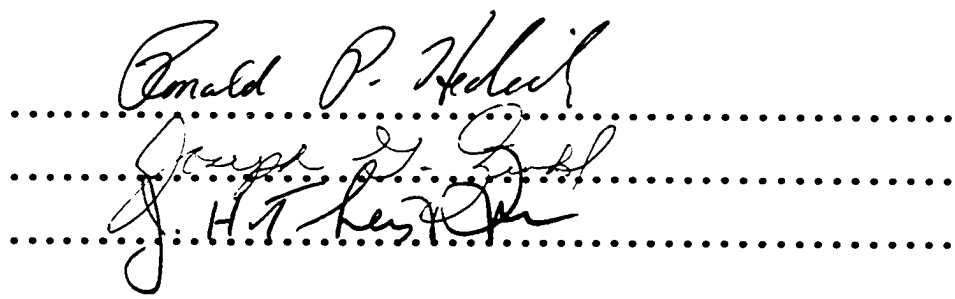

Committee in Charge

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Abstract

Rainbow trout, Oncorhynchus mykiss (formally Salmo gairdneri), which have recovered from proliferative kidney disease (PKD) were resistant to reinfection by natural exposure and to intraperitoneal (IP) injection of kidney homogenates which contained the parasite. The latter challenge method demonstrated that external defenses (skin, mucus components, gut enzymes, etc.) are not required for successful resistance to the parasite and suggests that an acquired immunity follows clinical PKD. In contrast, immunity did not develop in trout that had previous exposure to the infectious stage but that did not experience PKD.

In vitro incubation of PKX cells in serum from immune trout did not reduce experimental transmission suggesting that humoral factors alone do not confer resistance to infection.

The effect of subclinical PKD infection on blood parameters, disease resistance and immune defenses was examined in rainbow trout under laboratory conditions. Hematological and serological changes occurred primarily in those fish with severe renal lesions. Hematocrits decreased in the absence of signs of hemolytic anemia and leukocyte numbers, particularly phagocytes, increased in infected fish. Nephritis impaired divalent ion excretion and albumin

retention. There was a rise in several globular protein fractions (including immunoglobulin) in the plasma of infected fish. Infection by the PKX parasite stimulated the non-specific defenses of the trout as evidenced by the higher survival rate following challenge with the bacterial pathogen Vibrio anguillarum among PKX infected trout compared to control trout. Greater numbers of phagocytic cells were also present in the kidneys of trout with PKD compared to control fish. Chemiluminescence and nitroblue tetrazolium assays demonstrated that PKX infection did not reduce oxidative metabolism of activated, anterior kidney cells. PKX infection did not effect the efficacy of vaccination with V. anguillarum bacterin. Similar or heightened immune responses to sheep red blood cells as determined by plaque forming cell assays were also observed in trout with PKD in comparison to controls. It appears that subclinical PKD alone is not a major predisposing factor to secondary disease and affected fish can recover without serious problems if other environmental and biological stressors are reduced.

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Introduction

Proliferative kidney disease (PKD) is considered to be one of the most serious diseases among cultured salmonids in Europe and western regions of North America (Clifton-Hadley et al., 1984; Hedrick et al., 1986). A myxozoan parasite (PKX) with affinity to Sphaerospora sp. is the etiological agent for the disease (Kent & Hedrick, 1985b, Hedrick et al., 1988b). The intense inflammatory response to the parasite and the lack of complete sporogenesis of the parasite within the fish suggests that salmonids are unsuitable hosts for the PKX parasite (Kent & Hedrick, 1986).

While mortality attributed to PKD may exceed 90 %, most outbreaks produce a much lower mortality (Clifton-Hadley et al., 1984). High intensities of infection and considerable morbidity do not always correlate with high mortality (Hedrick et al., 1985). Affected fish can show decreases in food conversion, growth rate, tolerance to stress, and increased susceptibility to secondary pathogens (Seagrave et al., 1981; Clifton-Hadley et al., 1984). Economic impacts of PKD also include restriction by fishery authorities on fish planting from enzootic areas and facility quarantine. A better understanding of the host-parasite relationship is needed in order to develop biologically sound regulatory policies and management strategies (delayed exposure to

enzootic waters, etc.). These strategies should utilize information on the development of immunity, seasonality and severity of the disease in the salmonid host.

Ferguson & Ball (1979) reported that fish which had recovered from PKD did not develop clinical signs in a subsequent outbreak. The present study examined this resistance under laboratory conditions using two challenge methods. The contribution of humoral factors in survivor resistance was also evaluated in our study. The seasonal prevalence of the infectious stage of PKD was determined at the exposure site.

Host-parasite interactions can result in changes in the host's physiology and immune system. Many reports of PKD affected fish describe severe hematological changes and concurrent infections with other pathogens which may indicate impaired defense mechanisms (Hoffmann & Lommel, 1984; O'Brien et al., 1977; Ellis et al., 1982). The varying degrees of morbidity, mortality, and recovery reported prompted investigation into the isolated effects of subclinical PKD on the fish's blood parameters and immune status. Two challenge methods were employed in fish held under optimal laboratory conditions. Both non-specific and specific immune responses were examined in infected fish. Changes in selected hematological, serological, and immunological parameters were correlated with the degree of renal involvement during the disease.

The objectives of this study were to:

1) Determine the seasonality of the infectious stage of PKD and its relationship to the disease occurrence at the study's exposure site (American River hatchery).

2) Examine resistance to PKX reinfection by trout which had recovered from a previous infection.

3) Examine the effect of serum factors from PKD resistant trout on the infectivity of the PKX parasite and the possible role of these serum factors in resistance.

4) Examine the isolated effect of PKD on the fish's blood, serum, and immune parameters.

LITERATURE REVIEW

Geographic range. Proliferative kidney disease (PKD) in salmonid fishes is characterized as a granulomatous renal disease of protozoan etiology (Clifton-Hadley et al., 1984). The term "proliferative kidney disease" was first used by Roberts and Shepherd (1974) and has gained general acceptance. Seagrave et al. (1980a) referred to the parasite as Proliferative kidney disease organism "X" or PKX due to its uncertain taxonomy. The earliest account of this disease was by Plehn (1924) who attributed the pathological changes in the kidneys of brook trout (Salvelinus fontinalis, Mitchell) to an amoeba.

Proliferative kidney disease is a serious disease of farm-reared rainbow trout (Oncorhynchus mykiss, formerly Salmo gairdneri) in Europe (Clifton-Hadley et al., 1984). The disease has been found in salmonid fish in Italy, Germany, France, England, Ireland, Scotland, Sweden, Norway, and Denmark (Clifton-Hadley et al., 1984; Olesen & Jorgensen, 1986). The first North American outbreak was reported in 1981 by Smith et al. (1984) among rainbow trout at the Hagerman State Hatchery in Idaho. Proliferative kidney disease has subsequently been diagnosed in Pacific salmon and cultured rainbow trout in California, Washington, and Canada. Eight locations in California have been involved (Hedrick et al., 1985a), with the first outbreak in Pacific salmon and rainbow trout diagnosed at the California

Department of Fish and Game (CDFG) Mad River hatchery in 1983 (Hedrick et al., 1984). A review of histological slides of rainbow trout from the (CDFG) American River hatchery has revealed that a kidney condition previously called "lupus" was actually PKD from at least 1966 (Hedrick et al., 1985a). The disease was recently diagnosed in Pacific salmon from Washington state at culture facilities at Quinalt lake, Elwa river, and Turtle Rock (Hedrick et al., 1986a; pers. comm. K. Amos, Wash. Dept. Fish., Olympia). In Canada, PKD has been detected in Pacific salmon and steelhead trout (Oncorhynchus mykiss) at four sites in British Columbia and in Arctic char (Salvelinus alpinus) in Newfoundland (Hoskins & Kieser, 1986; Hicks & Ferguson, 1986).

Host range. Fish in the family Salmonidae appear to be the primary hosts affected by PKD but two non-salmonid species have also been reported to harbor the PKX organism. Seagrave et al. (1981) reported finding clinical and histological characteristics of PKD in wild yearling (1+) (Esox lucius) pike. Bucke et al. (1985) found the parasite in wild pike (Esox lucius) and roach (Rutilus rutilus). The majority of PKD reports involve cultured underyearling (0+) rainbow trout. Other salmonid species which have been reported to be infected include brown trout Salmo trutta, Atlantic salmon S. salar, Arctic char Salvelinus alpinus,

grayling Thymallus thymallus, coho salmon Oncorhynchus kisutch, and chinook salmon O. tshawytscha (Seagrave et al., 1981; Ellis et al., 1982; Bucke et al., 1985; Hicks & Ferguson, 1986; Hedrick et al., 1984). Recently, underyearling kokanee salmon (O. nerka) were found to be susceptible to experimental infections with PKX (Hedrick et al., 1987). It is questionable whether brook trout S. fontinalis are susceptible to PKD. Plehn (1924) reported PKD-like condition in this species, however in another report, Seagrave et al. (1981) observed that brook trout at an infected site did not contract PKD.

Parasite. Only recently has the taxonomic status of the PKX parasite been resolved. Early reports suggested that PKX was an amoeba, phylum Sacromastigophora, based on the parasite's formation of pseudopodia (Plehn, 1924; Ghittino et al., 1977; Ferguson & Adair, 1977). An amoeba isolated from rainbow trout with PKD-like signs by Ghittino et al. (1977) differed ultrastructurally from PKX in mitochondria shape, lack of inclusion cells, and the presence of microfilaments beneath the plasmalemma (Ferguson et al., 1978).

Seagrave et al. (1980a) suggested that PKX was a member of the Haplosporidia (phylum Acetospora) related to the oyster pathogens of the genus Marteilia. They based this assumption on ultrastructural and developmental similarities such as the presence of haplosporosomes, multivesicular

bodies, striated inclusions, and division by endogeny. The authors did not find any spore stages and therefore did not rule out the possibility that PKX might belong to the phylum Myxozoa, as members of this phylum demonstrate similar characteristics to those of the Acetospora. Myxozoans, unlike haplosporidans, are ubiquitous parasites of fish (Current & Janovy, 1977). An examination of PKX cells by electron microscopy revealed features consistent with myxozoan generative cells such as prominent Golgi apparatus, double membranes, cytoplasmic ribosomes, bundles of microtubules and envelopment of the tertiary cell by a secondary cell (Feist & Bucke, 1987).

Myxozoans are found principally as parasites of fish and their life cycle includes multicellular stages which differentiate during sporogenesis (Mitchell, 1977). Other characteristics of myxozoans include the cnidocysts or polar capsules present within a spore composed of valves. There may be one to six polar capsules within each spore, all of which contain a coiled filament. It is hypothesized that when extruded, the filament acts to anchor the spore to the host tissue (Mitchell, 1977). This group was recently separated from the unicellular phylum Protozoa and placed into its own phylum (Levine et al., 1980). A generalized lifecycle starts with the release of the amoeboid sporoplasm from the spore and its invasion of the host. This stage develops into a multicellular trophozoite which later

undergoes sporogonic development. The resting spore stage completes the cycle. Wolf & Markiw (1984) report a lifecycle for the myxozoan Myxobolus cerebralis, which includes an oligochaete as an intermediate host and an infective stage (Triactinomyxon sp.) for teleosts which is morphologically distinct from both later trophozoite and spore stages.

The observation of later sporogonic forms in convalescing fish gave strong evidence that PKX belongs in the phylum Myxozoa. Kent and Hedrick (1985b & 1986) observed myxozoan trophozoites and developing spores in the lumens of kidney tubules of PKD-infected fish. Although these intraluminal stages developed polar capsules, valve formation was incomplete. Further evidence that the intraluminal forms developed from the interstitial PKX stage was demonstrated when blood and spleen homogenates containing only interstitial PKX forms were injected into uninfected trout. These trout developed typical PKD with both interstitial and intraluminal stages (Kent & Hedrick, 1985a). The intraluminal sporogonic stages show similarities to Sphaerospora spp. (family Sphaerosporidae) in that both form pseudoplasmodia within the lumens of the kidney tubules, have small spherical polar capsules, and can be monosporous (Kent & Hedrick, 1986).

Hedrick et al. (1988b) observed spores and immature stages of a Sphaerospora sp. in the Tui chub (Gila bicolor)

from water directly above a hatchery with infected rainbow trout. The immature stages of the chub parasite closely resembled interstitial PKX, however, experimental cross-infection and co-habitation studies have not yet produced PKD in trout (unpublished data). Another similar myxosporean has been observed in sticklebacks (Gasterosteus aculeatus) from a PKD enzootic lake in Washington State (Hedrick et al., 1988b). This Sphaerospora sp. formed spores in the lumens of the kidney tubules and the early blood stage closely resembled PKX.

Parts of the life cycle of PKX, as it occurs in infected salmonids, has been described by Kent & Hedrick (1986). An as yet unknown infectious stage gains access to the fish's circulatory system and is hematogenously disseminated to various organs. The kidney is the principal site for vegetative development of PKX. Initially, PKX primary cells are found in the vascular spaces and are associated with the vessel wall. Later, these cells are found in the kidney interstitium, where they continue to multiply by binary fission, plasmotomy and endogenous daughter cell formation (Kent & Hedrick, 1986). A few interstitial PKX cells migrate between cells of the renal tubules, releasing their daughter cells into the lumen of the tubule. A daughter cell eventually acts as the enveloping cell or pseudoplasmodium of the six-cell spore (Lom et al., 1982). A majority of myxosporeans form multinucleated syncytia containing

sporoblasts called plasmodia (Mitchell, 1977). In contrast, the PKX sporoblast is monosporous and contains up to six cells which correspond to the two capsulogenic cells, two valvogenic cells and a binucleated sporoplasm (Kent & Hedrick, 1986). There has been no definitive observation of a PKX spore with complete valves although intraluminal forms have been observed up to 13 months post-infection (Foott & Hedrick, 1987). Hedrick et al. (1988b) report observing spores of a Sphaerospora sp. along with intraluminal stages identical to those of the PKX parasite in the kidney tubule of a rainbow trout held in PKD enzootic water. This trout had been exposed to the infectious stage of PKX in October when water temperatures were declining and was sampled the following April. The population from which the trout was taken did not demonstrate interstitial PKX infection in the monthly histological examination of kidneys from 30 fish. The urinary tract appears to be the exit route for the PKX spore if in fact any mature spores are produced.

The PKX parasite has been observed in the kidney, spleen, gut, pancreas, gill, liver, and muscle of heavily infected fish (Ferguson & Needham, 1978; Smith et al., 1984). Dissemination via the circulatory system is probably the route by which PKX reaches other organs. Parasites can be found in blood smears (Kent & Hedrick, 1985a). In hematoxylin and eosin stained sections from affected tissues, the PKX parasite is eosinophilic, measures 10-20 m

in diameter and has pseudopodia (Ferguson & Needham, 1978). The PKX parasite is often multicellular due to the presence of internal daughter cells and the nuclei contain a prominent endosome. There may be six to seven secondary and tertiary daughter cells within the primary cell. Ultrastructurally, the cytoplasm beneath the tri-lamellar plasmalemma of the primary cell contains numerous electron-dense inclusion bodies which measure 0.1 - 0.2 μm in diameter (Ferguson & Needham, 1978). These membrane-bound bodies have been called haplosporosomes by Seagrave et al. (1980a) and are characterized by an internal electron lucent bar. Secondary and tertiary cells have similar ultrastructural and organelle compositions as the primary cell, however, they do not contain haplosporosomes.

Wet mount examination of infected tissue by either brightfield or phase microscopy reveals prominent refractile granules adjacent to the plasmalemma. These granules are most likely involved in glycogen storage as they stain positive in periodic acid Schiff staining (Ferguson & Needham, 1978).

The interstitial PKX parasite reaches the lumen of the kidney tubules and then undergoes sporogonic development. Kent & Hedrick (1987) reported that reducing the inflammatory response with cortisol implants resulted in higher densities of interstitial PKX and subsequent transformations to the intraluminal stages compared to

normal infections. The most simple intraluminal form observed is the uninucleate secondary cell which has recently been released from the primary cell. Later development through endogeny produces a multinucleated sporoblast enveloped by the original secondary cell (pseudoplasmodium). The sporoblast measures 12 by 7 μm and can contain up to six nuclei within the pseudoplasmodium (Kent & Hedrick, 1986). The two spherical polar capsules measure 2 μm in diameter and stain intensely with Price's Giemsa. The intraluminal stages have not been observed to be associated with host macrophages unlike the interstitial PKX stages which invoke a strong inflammatory response.

Epidemiology. The nature of the infectious stage of PKX has not been documented. Transmission of PKX by intraperitoneal injection of infected blood, kidney or spleen suspensions containing PKX or exposure to water containing the infectious stage are the only methods for the induction of experimental infections (D'Silva et al., 1984; Kent & Hedrick, 1985a). Foott et al. (1986) reported that exposures to a water supply (presumed to contain the infectious stage) for as short as 3 d can result in infection. Attempts at infecting salmonids by feeding kidneys containing PKX parasites have been unsuccessful (D'Silva et al., 1984). Whether infected salmonids can transmit the parasite to another host is still unclear.

Although salmonids are considered to be abnormal hosts for PKX based on incomplete spore development and the intense host response, transmission from an infected salmonid to a natural host may be possible (Hedrick et al., 1986b).

The route of entry for the infectious stage in natural infections is unknown. The PKX parasite has been observed in both rainbow trout intestinal mucosa and in the gill lamellae (Smith et al., 1984; Hedrick et al., 1984) but these stages may be a result of dissemination via the circulation to these tissues rather than points of entry.

There is a wide variation in the severity of PKD outbreaks (Clifton-Hadley et al., 1984). While mortalities of 95% have been reported in affected populations (Seagrave & Bucke, 1979; Hedrick et al., 1984), most outbreaks produce a much lower mortality. High numbers of parasites and morbidity do not always correlate with high mortality (Hedrick et al., 1985a). Clifton-Hadley et al. (1987a) concluded that severe renal swelling precedes typical clinical signs. Other biological and environmental factors appear to exert a greater influence on mortality. Concurrent infections of such pathogens as Flexibacter columnaris, Ichthyophthirus multifilis, Ichtyobodo necatrix, Sanguinicola sp., infectious pancreatic necrosis virus, infectious hematopoietic necrosis virus, fungal infections and disease conditions such as nephrocalcinosis are often associated with PKD mortalities (O'Brien et al., 1977;

Hoffmann & Dangschat, 1981; Ellis et al., 1982; Smith et al., 1984). The chronic anemia which may occur in affected fish increases the severity of such stress situations as low dissolved oxygen levels, sorting, and high loading densities (Clifton-Hadley et al., 1984). Soft, acidic water has been considered a predisposing factor to PKD, however, outbreaks also occur in hard, alkaline waters (Ferguson & Needham, 1978; Scott, 1979; Hedrick et al., 1984). Species and strain susceptibility may also influence severity of the disease. Ellis et al. (1985) reported that certain native Atlantic salmon stocks were resistant to clinical disease in comparison to imported Norwegian stocks. Differences in the prevalence, intensity, and lesions of PKD infections among Pacific salmonids has been documented by Hedrick et al. (1987). They found that experimentally infected chinook and kokanee salmon may have greater numbers of parasites within the kidney interstitium and a lesser degree of host response than rainbow trout and coho salmon.

Proliferative kidney disease outbreaks typically occur in underyearling fish, however, older fish are also susceptible to infection (D'Silva et al., 1984; Clifton-Hadley et al., 1984). In most locations, PKD outbreaks are seasonal and occur primarily from May to November when water temperatures are 15 C or above (Ferguson & Ball, 1979; Clifton-Hadley et al., 1984). Foott & Hedrick (1987) demonstrated that the seasonality of PKD at one location corresponded to the

period when the infectious stage was present in the water supply and not directly to the water temperature. The effect of temperature on the infectious stage has not been documented. Experimental infections have been produced at 7 and 11 C but development of the disease was slower when compared to infections at 17 C (Rafferty et al., 1985). Ferguson (1981) reported that declining water temperatures correlate with a reduction in both morbidity and mortality. It appears that the primary effect of water temperature is on the progress of the disease and not on the initial stages of infection.

Proliferative kidney disease not only affects fish production by increased stock mortality but also by reductions in growth, food conversion, and resistance to handling stress (Clifton-Hadley et al., 1984). A number of control measures have been attempted with varying results. There are few reports of chemotherapeutic compounds which have been successful in controlling PKD (Ghittino et al., 1977; Ferguson & Ball, 1979; Bucke et al., 1981). Recently, a successful prophylaxis was induced by feeding the antiprotozoan drug Fumagillin (Chinoin, Budapest, Hungary) at a dosage level of 0.05 g/kg food (Hedrick et al., 1988). Clifton-Hadley & Alderman (1987) report that multiple, high concentration flush treatments of malachite green had a therapeutic effect on PKD-infected fish, however, acute toxicity and high tissue concentrations of malachite green

were a problem. Manipulation of culture conditions is currently the only viable management method for PKD. Ferguson & Ball (1979) reported that delaying the stocking of fish into water supplies containing the infectious stage from May until July resulted in a decrease of disease severity. Reduction of hatchery water temperature has also been successfully employed to reduce PKD severity (Hoskin & Kieser, 1986). O'Hara (1985) observed that transfer to saltwater reduced mortality in affected Atlantic salmon but Hedrick & Aronstein (1987) demonstrated that the progress of PKD in Pacific salmon was not impeded by saltwater transfer. They concluded that the major benefit of transfer to saltwater may be the reduction of external freshwater pathogens. Removal of the infectious stage by ultraviolet treatment of the water supply has been shown to be an effective prevention method (Hedrick et al., 1985a). Other myxosporeans such as Myxobolus cerebralis and Ceratomyxa shasta have been similarly controlled by water treatments (Hoffman, 1975; Sanders et al., 1972). As few therapeutic measures are available at this time, limiting exposure to the infectious stage is the primary management scheme. Seagrave et al. (1981) stated that secondary disease and husbandry-related stress are contributing factors to mortality during PKD epizootics.

Pathology. Similar chronological patterns of the disease has been described by several workers (D'Silva et al., 1984;

Clifton-Hadley et al., 1985; Kent & Hedrick, 1986). The parasites are first detected microscopically in the kidney 3 - 5 wk after exposure to infectious stage or following intraperitoneal injection of homogenates of kidneys from infected fish. The numbers of interstitial PKX parasites increases to a peak at 7- 12 wk post-exposure and gradually diminishes along with the resultant interstitial inflammation. Intraluminal forms are first observed at 5 wk post-exposure and may persist for at least 13 mo (Foott & Hedrick, 1987).

Affected fish may have a number of non-specific external signs which including lateral body swelling due to kidney hypertrophy, darkened body color, bilateral exophthalmia, abdominal distention from ascitic fluid, and pale gills due to anemia (Ferguson & Needham, 1978; Clifton-Hadley et al., 1984; Hedrick et al., 1984). Kidney swelling without overt external signs has been reported (Kent & Hedrick, 1986). Internally, the most prominent signs are kidney and spleen enlargement. The kidney may have a grey mottled pattern due to granulomatous lesions and in severe cases the capsule has a corrugated appearance. These signs can mimic those of viral hemorrhagic septicemia, bacterial kidney disease, and infection by the fungal pathogen Ichthyophonus hoferi (Clifton-Hadley et al., 1984; Hedrick et al., 1986a).

Microscopic examination reveals progressive lesions. Initially, the PKX parasite invades the vascular spaces of

the kidney and vasculitis may result (Smith et al., 1984). Further PKX multiplication and invasion of the kidney interstitium produces hyperplasia of the surrounding hematopoietic cells. As the disease progresses, macrophage and lymphocyte infiltration occurs leading to diffuse granulomatous lesions (Clifton- Hadley et al., 1985; Ferguson & Needham, 1978). Pressure atrophy and displacement due to interstitial hyperplasia causes tubular degeneration and an reduction in the number of tubules/mm^2 (Kent, 1985). Eosinophilic hyaline droplets are found in epithelial cells of the proximal tubule, perhaps implying tubular disfunction (Clifton-Hadley et al., 1987b). The lesions gradually resolve and the kidney returns to a normal histological appearance by 12 - 20 wk post-exposure (Clifton-Hadley et al., 1985).

Proliferative kidney disease can affect several hematological and serological parameters. The anemia associated with PKD has been described as chronic and hemolytic based on the presence of intact erythropoiesis regions, increases in immature RBCs, and increased hemosiderin deposition in the spleen (Hoffmann & Lommel, 1984). Hemoglobin crystals have been observed in kidneys of affected fish (Clifton-Hadley et al., 1987a). Clifton-Hadley et al. (1985) speculated that the granulomatous lesions may impair the transport of newly-formed red blood cells. Ferguson & Needham (1978) reported an average red

blood cell (RBC) count of 0.38 x 10^6 and an average hematocrit of 11.2 % in fish with clinical signs of PKD. In comparison, Wedemeyer & Yasutake (1977) give the reference ranges for trout RBC counts as 0.77 - 1.58 x 10^6 and hematocrit as 24 - 43 %. Ghittino et al. (1977) reported a leukocytosis in affected fish, however, they made their observation from blood smears and not white blood cell (WBC) counts. Hoffmann & Lommel (1984) reported no major change in the leukocyte count or leukocyte population composition in PKD affected fish. Clifton-Hadley et al. (1987a) reported that the number of immature lymphocytes in the blood increases as the disease progresses. Clifton-Hadley et al. (1987b) also stated that infected fish showed a neutrophilia.

The hypoproteinemia commonly observed appears to result from the reduction of the albumin fraction (Scott, 1984; Klontz et al., 1986). Hypoalbuminemia is probably related to the increased permeability to low molecular weight proteins of damaged glomeruli, reduced reabsorption function of damaged proximal tubules, and overall reduction in functional nephrons rather than impaired liver biosynthesis. Scott (1984) reported that a non-immunoglobulin fraction "F4" rose in trout surviving PKD infections and exposed to the infectious stage a second time. He suggested that "F4" may be an acute phase protein. In comparison to the reduction in total serum protein, hypergammaglobulinemia is

reported in clinically ill trout. Olesen & Jorgensen (1986) found an average IgM concentration of 10.9 ± 5.3 mg/ml which constituted 34 % of the total protein in comparison to 5 - 6 % in normal trout. Klontz et al. (1986) also reported a rise in the globulin fraction of sera from affected fish. It is unknown if this rise in immunoglobulin represents a specific defensive response to PKX antigens.

Ferguson & Ball (1979) observed that trout which survived clinical PKD did not show any clinical signs of the disease in subsequent outbreaks. Cell-mediated immunity against the PKX parasite may be a major mechanism for the observed protection. The PKX parasite is often surrounded by host macrophages and lymphocytes (Ferguson & Needham, 1978). These granulomatous lesions are similar to delayed hypersensitivity lesions in mammals (Ellis et al., 1985). Angelidis et al. (1987) reported that trout with gross PKD lesions in the kidney had higher mortality from an intraperitoneal (IP) challenge of Vibrio anguillarum than cohorts without gross lesions. These fish were immunized by IP injections of a Vibrio bacterin 15 d prior to the challenge. The degree of kidney swelling did not affect the anti-V. anguillarum serum agglutination titer. Anterior kidney cells from trout with gross lesions had lower chemiluminescence responses than cells from cohorts without gross lesions.

The immune system appears to play a central role in

both the pathogenesis of PKD and the development of disease resistance seen in recovered fish. Yet to be understood are the relationships of humoral, non-specific, and cellular defenses in resistance to reinfection. Stress can have a major impact on the immune system of fish (Peters et al., 1988). In order to more fully understand the host-parasite relationship which occurs in PKD, both the stress response and the immunodefense system of the infected fish must be examined during the course of the disease.

SALMONID DEFENSE MECHANISMS

Fish have a number of mechanisms which aid in protection against invasion and infection with foreign agents. These mechanisms fall under two broad categories, nonspecific and specific.

Nonspecific defense mechanisms can be induced by many unrelated, non-antigenic agents and characteristically there is no memory as seen with specific mechanisms (Ellis, 1981). Components of nonspecific defense include the epidermis and mucus layer which act as barriers to invasion of foreign agents. Pickering (1977) demonstrated that seasonal changes in thickness of the epidermis and mucus cell distribution resulted in increased susceptibility to skin infections. The non-keratinized epidermis heals rapidly reflecting the teleost's requirement for maintaining osmotic integrity (Roberts, 1978). Mucus acts to inhibit colonization of the

fish's integument and contains lysozyme, complement proteins, C-reactive protein, proteases and immunoglobulins (Fletcher & White, 1973; Ramos & Smith, 1978a; Hjelmeland et al., 1983; Harrel et al., 1976). The immunoglobulin present in the mucus is secreted by lymphocytes located in the dermis (St.Louis-Cormier et al., 1984). Extremes in pH and the presence of digestive enzymes provide the gastro-intestinal tract with a defense against ingested microorganisms.

Salmonid sera normally contains a higher level of acute phase proteins in comparison to mammals. One such component is C-reactive protein which reacts with the phosphoryl choline residues of bacterial cell walls and causes bacterial agglutination (Winkelhake & Chang, 1982). Leukocyte-produced lysozyme is normally present in the serum although its concentration is influenced by immunogenic stimulation and the season (Vladimirov, 1968). Lie et al. (1989) describe two lysozyme variants in the kidneys of rainbow trout. In higher vertebrates, lysozymes are involved in bacteriolysis, opsonization, and carbohydrate digestive functions (Jolles & Jolles, 1984). Alexander & Al-Shakerchi (1986) have described a protein in Atlantic salmon serum which has an alpha-mobility and affinities for fungi (Saprolegnia) and "diseased salmon tissue". Another alpha-migrating protein was found to have an anti-protease activity similar to mammalian alpha-2 macroglobulin (Ellis,

1981). Serum may also contain interferon which has antiviral effects (De Kinkelin et al., 1982).

The complement components can be activated in either a non-specific (alternate system) or antibody-mediated fashion (classical system). Salmonids are reported to possess both systems (Nonaka et al., 1981; Sakai, 1981).

Phagocytes provide teleosts with a temperature-insensitive defense mechanism which can deal with invading foreign objects, aid in the immune reaction, and help resolve tissue damage (Finn & Nielson, 1971; Johnson & Smith, 1984). In salmonids, cell types shown to phagocytize particulate matter include the gill pilar cells, neutrophils, monocytes, macrophages and endothelial cells lining blood sinuses (Goldes et al., 1986; MacArthur & Fletcher, 1985). The literature contains conflicting reports on the phagocytic ability of teleost neutrophils, however, when salmonid neutrophils are mature and activated they are phagocytic (Finn & Nielson, 1971; Griffin, 1983). Salmonids do not have discrete melanomacrophage centers as do many teleosts but rather have pigmented macrophages randomly distributed throughout lymphoid tissues (Aguis, 1979). These mononuclear phagocytes are involved in iron-storage as well as phagocytosis. A histaminogenic cell type has been described in salmonids. Ellis (1985) reports that the eosinophilic granular cells found in the gut mucosa will respond non-specifically to Aeromonas salmonicida

extracellular products by releasing a histamine-like compound which may initiate inflammation. Inflammation serves as a link between non-specific and specific defenses.

The specific immune response is comprised of humoral and cell-mediated processes. O'Leary (1980) reported that rainbow trout have both a high molecular weight tetrameric (620,000 daltons) and a lower molecular weight trimer (490,000 daltons) immunoglobulin. Salmonid immunoglobulin displays a beta-2 to gamma-1 electrophoretic mobility and normally accounts for 5 - 6 % of the total serum proteins (Ingram & Alexander, 1980; Olesen & Jorgensen, 1986). Antigen-specificity of the immune response has been demonstrated in several hapten-carrier studies with salmonids (Anderson et al., 1982; Kaattari et al., 1986). Antigen tolerance can be induced in rainbow trout (Sakai, 1984a). An accelerated secondary response occurs in fish, however, water temperature and the type of antigen influences the memory response (O'Neil 1980 ; Anderson et al., 1982).

Low water temperature can delay or even abolish protective immunity in salmonids (Fryer et al., 1976). The primary immune response to bacterial antigens occurs 14 - 16 d post-injection in salmonids maintained at 12 - 16 C (Anderson et al., 1979b; Kaattari & Irwin, 1985). Seasonal modulation of the immune response independent of water temperature has been documented in rainbow trout. Yamaguchi

et al. (1980) reported that rainbow trout maintained at 18 ± 1 C and immunized prior to spring, had a greater antibody response than trout immunized in the fall.

The three lymphoid organs involved in the immune response are the pronephros, spleen, and thymus. A mammalian nomenclature of central and peripheral lymphoid tissue is not relevant to fish as immunoglobulin-secreting cells are present in all three organs (Kaattari & Irwin, 1985). The pronephros performs the functions of hematopoiesis, filtration of foreign substances, and is a primary lymphoid organ (Zapata, 1979; Bayne, 1986). Lymphocytes of the pronephros tend to be more restricted in their responses to mitogens and antigens than splenic or blood lymphocytes (Kaattari & Irwin, 1985; Etlinger et al., 1976). This difference in responsiveness may indicate that pronephros lymphocytes are more immature than those found in the spleen or blood. They may be similar to mammalian bone marrow lymphocytes. The salmonid spleen performs filtration and lymphocyte maturation functions but does not have distinct red and white pulp zones as do certain other fish (Anderson, 1974). Chilmonczyk (1983) described the trout thymus as having three zones covered by a thin, perforated epithelium. In situ labeling has shown that thymocytes migrate to the spleen and kidney (Tatner, 1985).

Whether lymphocyte heterogeneity exists in salmonid fishes is not completely clear. Functional assays such as

mixed lymphocyte reactions, mitogen response, and hapten-carrier specificity suggest that salmonids possess a lymphocyte population analogous to murine T-cells (Etlinger et al., 1977; Etlinger et al., 1976; Kaattari et al., 1986). The application of murine T and B cell nomenclature to salmonids is tenuous however, as all rainbow trout lymphocytes, including thymocytes, have membrane-bound immunoglobulin (Warr et al., 1979).

Several workers have demonstrated lymphokine-like production in salmonids and the importance of monocyte/lymphocyte interaction in the immune response in fish (Smith & Braun-Nesje, 1982; Blazer et al., 1984; Clem et al., 1985). In addition, a cell functionally analogous to the mammalian natural killer (NK) cell has been described in salmonids (Moody et al., 1985).

In summary, the salmonids possess a defense system with many of the same components found in higher vertebrates. One important difference is the effect temperature has on the performance of the immune system of these poikilotherms. Stress can also have a major impact on the immune capabilities of fish and their susceptibility to various pathogens (Wedemeyer, 1970a).

Stress and Health Assessment. The concept of stress and the terminology used to describe this phenomenon has been evolving for many decades with many authors incorrectly

using the term stress to represent both the stimulus and the response. Seyle (1950) defined stress as "the sum of all the physiological responses by which an animal tries to maintain or re-establish a normal metabolism in the face of a physical or chemical force". In the "General Adaptation Syndrome", the stress response is chronologically divided into three possible stages: 1) an alarm reaction, 2) physiological resistance and adaptation, and 3) exhaustion (Seyle, 1973). Schreck & Lorz (1978) demonstrated that fish must perceive pain or fright in order to undergo a stress response. In their experiments, coho salmon (Oncorhynchus kisutch) did not exhibit stress when exposed to lethal doses of cadmium in comparison to a dose-related stress response to the more noxious metal copper. The effects of stress are manifested at primary, secondary, and tertiary levels. The primary level of the response is neuro-hormonal in nature with catecholamines (CA) and corticosteroids (CS) as mediators. In salmonids, epinephrine is the major catecholamine released by the chromaffin tissue of the anterior kidney (Mazeaud et al., 1977). Smaller amounts of CA are also liberated by sympathetic nerve endings. The release of CA occurs within seconds of the stress stimulus and results in the secondary level effects of tachycardia, hyperglycemia, increased gill permeability, hypercholesteremia, and splenic contraction (Mazeaud & Mazeaud, 1981). The major corticosteroid (CS) of salmonids

is cortisol which is released within an hour of the stress stimuli. Nerve stimulus of the hypothalamus causes corticotropin release factor (CRF) to be transported to the pituitary which in turn causes release of adrenocorticotropic hormone (ACTH). ACTH stimulates the interrenal cells of the kidney to excrete cortisol. Corticosteroid release is slower than that of the CAs and can continue for many days. The secondary effects of CS release into the bloodstream include a shift to protein catabolism for gluconeogenesis, increased sodium loss from the gills, reduced phagocytic cell response and leukopenia (Turner & Bagnara, 1971; McLeay, 1973; Stave & Roberson, 1985). Lymphopenia in some mammals has been attributed to a lympholytic action of CS (Burton & Murray, 1979). Lysosome membrane fragility due to CS is one mechanism proposed for leukopenia (Smith, 1982). Maule et al. (1987) reported that exogenous cortisol did not cause lympholysis but may have resulted in a reduction of the B-cell subpopulation. Kaattari & Tripp (1987) showed that antibody response suppressed by cortisol could be restored with the addition of conditioned media which was presumed to contain macrophage interleukin(s). A tertiary effect of a cortisol-related reduction in leukocyte activity is an increase in disease susceptibility (Pickering & Duston,1983). Other tertiary effects of CS may include changes in behavior, growth, smoltification and decreased spawning success

(Wedemeyer & McLeay, 1981). The duration of contact with the stressor has a major impact on the tertiary level effects. Chronic stress, in which adaptation is not accomplished, is implicated as a major factor in the susceptibility of fish to disease (Wedemeyer, 1970a).

Over the last two decades, there has been increased effort to elucidate the physiological responses in fish to stress and disease. Blood and mucus parameters have been studied for use as health indicators. Mucus levels of occult hemoglobin, ketones, CRP, and cellularity have been identified as indicators of husbandry-induced stress (Ramos & Smith, 1978a,1978b, Smith & Ramos, 1976, Wechsler, 1984). Arillo et al. (1979) reported that the gill sialic acid (a major component of mucus) increases when fish are exposed to levels exceeding 0.02 ppm unionized ammonia. Blood indices such as hemoglobin content, hematocrit (Hct), leukocrit (Lct), differential counts, coagulation rate, and erythrocyte sedimentation rate (ESR) have also been investigated as indicators of general health (Wedemeyer & Yasutake, 1977; McLeay & Gordon, 1977; Casillas & Smith, 1977; Barham et al., 1980). Of these parameters coagulation rate, ESR, and Lct appear to be the best early indicators of poor health. Wedemeyer et al. (1983) cautioned that Lct was a sensitive indicator of acute stress following handling but showed poor correlation to chronic stress situations or as a diagnostic index for sub-clinical bacterial infections.

Serological parameters such as glucose, cholesterol, enzymes, complement, chloride, calcium, and cortisol have been utilized for health indicators. Induced stress can result in hyperglycemia, hypocholesterolemia and lack of calcium and chloride ion control in salmonids (Wedemeyer, 1973; Wedemeyer & Yasutake, 1974). Sakai (1983) demonstrated that the activity of the alternative complement system as measured by foreign RBC hemolysis (SH_{50}) was reduced in proportion to the disease state of the salmonid. Several workers have documented disease-related changes in such enzymes as glutamic oxalacetic transaminase and lactic dehydrogenase (Bell, 1968; Amend & Smith, 1974). Numerous workers have directly measured plasma cortisol levels as a primary level stress effect (Fagerlund, 1967; Donaldson & Dye, 1975; Barton et al., 1980). Wedemeyer & Yasutake (1977) used interrenal tissue ascorbic acid depletion as an indicator of corticosteroid production in salmonids. Microscopic tissue examination has been employed as a health assessment tool. McLeay (1975) examined interrenal cell hypertrophy as a measure of chronic stress. Peters & Schwarzer (1985) demonstrated that blastogenesis decreased as both precursor cells and immature leukocytes of the anterior kidney convert to a macrophage-like cell during periods of stress. They speculated that the initial response aided the fish by increasing phagocytic capability, however due to reduced blastogenesis, chronic stress would result in

leukopenia. More work will be needed to define the limitation of each of these indicators and to bring the level of fish health assessment to those of mammalian medicine.

MATERIALS & METHODS

Fish maintenance

All aquaria at the UC Davis Fish Disease Wet Laboratory (UCD-FDL) were supplied with temperature-controlled, well water. The water hardness and dissolved oxygen (D.O.) of the intake water was 160-170 ppm $CaCO_3$ and 9 ppm O_2 respectively (Hach kit measurements). Two types of flow-thrcugh aquaria types were used: rectangular 130 L tanks and circular 650 L tanks. Both types were supplied with airstones. Fish were fed daily on a diet of dry trout pellets (Silvercup) at a rate of 2 % body weight/day. Anesthesia was performed with MS-222 (Sandoz) at either 50 ppm for injections or 65 ppm for bleeding and sacrifice. It was not necessary to buffer the anesthetic solution as the water hardness maintained the pH at 7.2 (Wedemeyer, 1970b).

Fish

Rainbow trout used in the following studies were provided by the California Department of Fish and Game. These fish came from either American River hatchery (Rancho Cordova), Darrah Springs hatchery (Red Bluff), or Moccasin Creek hatchery (Don Pedro reservoir). Five strains of rainbow trout were utilized; Eagle Lake, Shasta, Shasta/Kamloops cross, Hot Creek, and Coleman (Table 1).

Histology

Stained tissue samples were used to detect the presence of stages of PKX and to assess renal lesions.

Table 1. Abbreviations for hatcheries and fish stocks used.

California Department of Fish and Game (CDFG) Facilities	
American River Hatchery Rancho Cordova, California	AR
Darrah Springs Hatchery Red Bluff, California	DS
Moccasin Creek Hatchery Don Pedro Reservoir, California	MC
Rainbow trout stocks (as denoted by CDFG)	
Eagle Lake	ELT
Shasta	RTS
Shasta/Kamloops cross	RTS/RTKJ
Hot Creek	RTH
Coleman	RTC

Tissue samples were fixed for 24 h in Davidson's solution (Humason, 1979) and changed to 50 % ethyl alcohol for storage. The samples were embedded in paraffin, sectioned at 6 μm and stained with Harris hematoxylin and eosin Y. The extent of lesions (LESION) and intensity of infection for both interstitial (PKX) and intraluminal (LUM) parasites were rated on each kidney section. A scale of 0 (none) to 4 (high) in increments of 1 were awarded for each of the above categories. Fish were grouped into four classifications according to these ratings (Figs. 1-4):

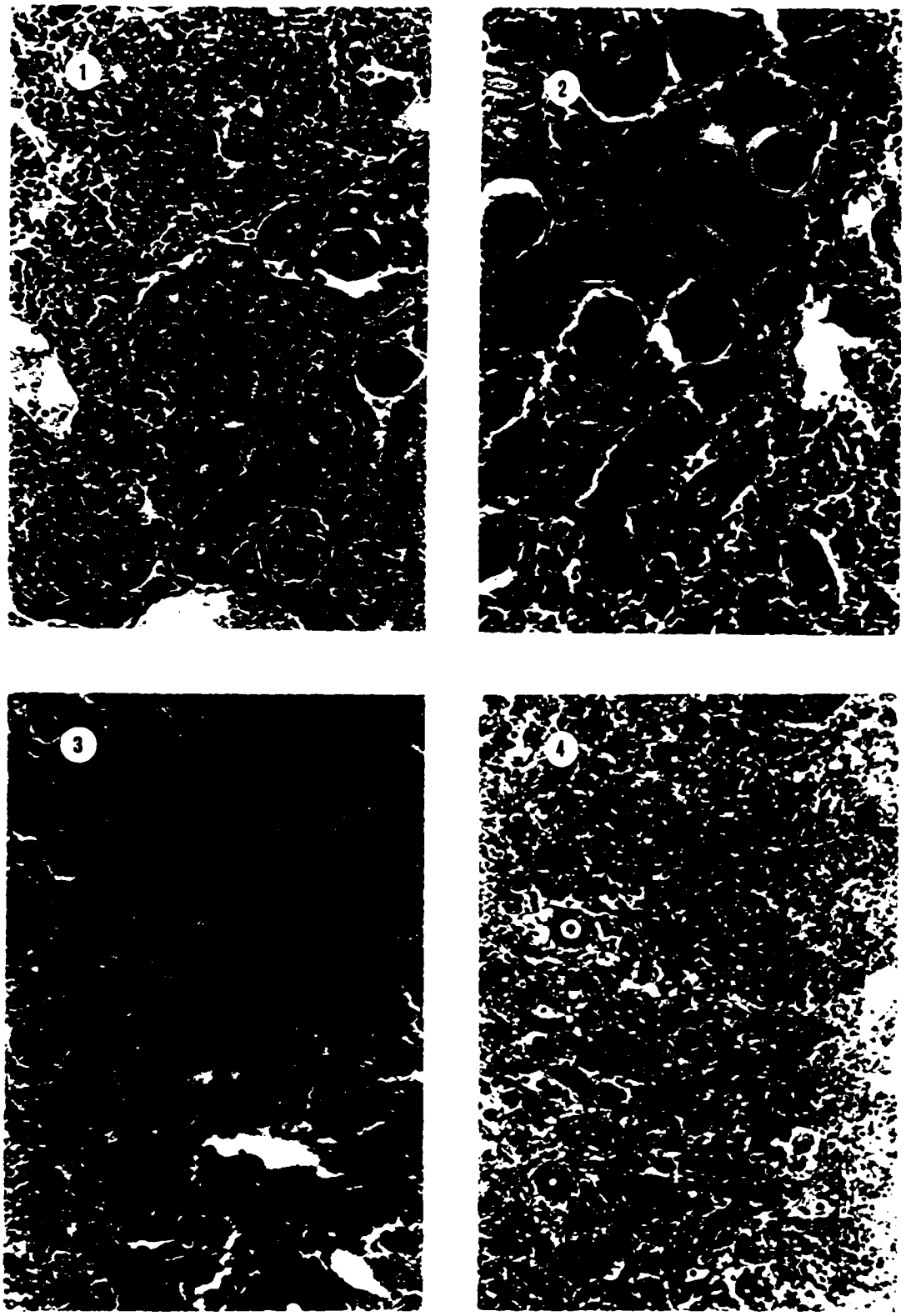
Normal	(0)	no PKX or LESION
Light	(L)	light PKX infection with or without focal lesions PKX 1 LESION 0-1
Moderate	(M)	moderate PKX infection and kidney lesions PKX 2 LESION 2
Heavy	(H)	heavy PKX infection and diffuse kidney lesions PKX >2 LESION >2

This scheme was chosen to divide the infected fish into disease severity groups rather than use the chronological sample date as the sole determinant. At each sample period there were a few individuals which showed marked differences in renal lesions compared to a majority of the same sample.

Survivor resistance: natural infection

Resistance to PKX reinfection and disease was examined in trout which had recovered from PKD and were challenged again by either field exposure or intraperitoneal injection. Field challenges occurred at the California Department of Fish

Figure 1-4. Posterior kidney sections (6 μm) of rainbow trout, Oncorhynchus mykiss, illustrating the disease rating categories. Arrow = PKX parasites. H & E. Bar = 50 μm. 1. Posterior kidney of control trout (PKD - 0). 2. Early, light infection (PKD - L). 3. Beginning of resolution stage in which few interstitial parasites are present, moderate infection (PKD - M). 4. At peak of disease, heavy infection (PKD - H).



and Game American River hatchery (ARH), Rancho Cordova, California. Proliferative kidney disease has been enzootic to the hatchery since at least 1966 (Hedrick et al., 1985a). The water supply for this facility is taken directly from Lake Natomas just above the hatchery. In order to determine the period(s) when the infectious stage of PKD was present in the hatchery water supply, a sentinel trout study was conducted monthly from September 1984 through October 1985. Groups of 20 - 35 0+ rainbow trout from a hatchery free of PKD were exposed to water at ARH in 38 L aquaria for 4 wk. The fish were then transferred to the UC Davis wet laboratory and held in 18 C well water for an additional 4 wk in 15 L aquaria. Eight weeks from the initial exposure, the remaining fish were sacrificed and the kidney tissues were processed for microscopic examination. An additional group of 20 - 35 fish were exposed to ARH water heated to 17 C from November 1984 through May 1985. These fish were exposed and sampled in parallel to those in ambient water.

Three production populations at ARH, designated groups A, B, and C, were sampled monthly to determine the effect that time of introduction to an enzootic water supply had on disease resistance and to provide prevalence of infection data for trout used in the survivor resistance study. Kidney sections of 30 fish from each group were microscopically examined for PKX stages and renal lesions. Groups A, B, and C were brought to the hatchery on August 16, October 12, and

December 3, 1984 respectively. Fish (18 mo) from group A which had an average weight of 630 g (July 1985) served as the experimental group during the 1985 natural exposure study. The controls were 17 mo old trout (avg wt 733 g) from Moccasin Creek hatchery, a facility free of PKD. Fifty fish from each group were held in two 650 L circular tanks and exposed to ARH water from June 16, 1985 until sampled 8 wk later, on August 13, 1985 for histological examination.

Survivor resistance: Experimental infection

Artificial challenges were conducted to examine the effect of an abnormal route of parasite invasion on resistance to reinfection. The survivor fish consisted of yearling (ELT85) rainbow trout (avg wt 138.4 g) which had undergone a PKD outbreak at ARH in the summer of 1985. The unexposed yearling (RTS/RTKJ85) trout were from Darrah Springs hatchery (DS), a PKD-free facility, and had an average weight of 147.6 g. Both the survivor and unexposed trout were divided into two groups of 28 fish, one of which received injections of PKX and the other an equal volume of Eagle's minimum essential media (MEM). The groups were maintained in 130 L tanks at 16 - 17 C.

A PKX inoculum was obtained from infected rainbow trout (avg wt 10.3 g). Kidneys were surveyed for the presence of parasites by wet mount examination, and those with high concentrations of parasites were excised and placed in MEM

on ice. Fifteen infected kidneys were teased through a wire screen and pooled in 12 ml of MEM. Trout in the experimental group received a 0.2 ml intraperitoneal (IP) injection of this kidney suspension while control fish were injected with an equal volume of MEM. At 2 wk intervals, five fish from each of the four groups were anesthetized in 55 ppm MS-222 (pH 7.0) and bled from the caudal vein with a 22 Ga. needle on a 5 ml vacutainer tube (Monoject). The blood was allowed to clot overnight at 4 C, centrifuged, and the sera from five fish was pooled and frozen at -80 C. This serum was analyzed for chloride, calcium, albumin, and total protein concentrations. Serum chloride was determined by the mercuric thiocyanate method (Sigma procedure 460), serum calcium by the O-cresolphthalein complexone method (Sigma procedure 586), albumin by the bromcresol green method (Sigma procedure 631), and total protein by a triple scale refractometer (Reichart A/O). The study began on September 16, 1986 and ended 8 wk later with the sacrifice of all groups on November 17, 1986. The fork length, weight, hematocrit and leukocrit were recorded for each fish. A blood smear was also taken and the kidney was fixed in Davidson's solution and later processed for histological examination.

Effect of serum from survivor trout on parasite infectivity

In vitro incubation of PKX parasites with sera from PKD-

recovered trout prior to IP injection into recipient fish was conducted to determine if humoral factors in survivor sera would affect experimental transmission. Three groups of 35 rainbow trout, avg wt 23.1 g (RTC strain), were injected IP with 0.1 ml of one of the three solutions described below. An additional group of 35 fish, injected with saline, served as negative controls. All four groups were maintained in separate 130 L aquaria receiving 16 C well water.

An infected stock of rainbow trout (15 - 20 g) was surveyed for the presence of PKX parasites by examination of fresh mounts of the kidneys. The kidney and spleen from six fish with large numbers of parasites were teased through a wire screen and pooled in 10 ml 0.85 % saline. The parasite suspension was divided into three equal aliqouts of 3 ml each and incubated for 30 min at 25 C with one of the following solutions: a) serum of five fish from a population which had recovered from a previous outbreak of PKD (no histological signs of interstitial PKX when sampled at the time of bleeding), b) control serum from 10 yearling trout from a hatchery with no history of PKD or c) saline. Each mixture was composed of one part saline or serum and two parts parasite suspension. The study began on February 14 and ended on April 3, 1986 when all groups were euthanized and kidney tissues examined for PKX stages and renal lesions.

Physiological & Immunological Parameters:

Experimental infection

The effect of experimental PKX infection on blood and immune system parameters of trout was examined in the following studies. Three hundred and fifty yearling trout (avg wt 106.4 g) from a hatchery with no history of PKD (Darrah Springs) were each injected IP with a 0.2 ml suspension containing PKX parasites. Another 350 trout from the same stock were injected IP with 0.2 ml of MEM. The groups were held in separate 650 L circular tanks supplied with 16 C well water. Loading density was reduced 5 d later when 210 fish from each group were moved to a series of 130 L tanks for other experiments.

Fifty trout kidneys with numerous parasites were teased through a wire screen and pooled into 90 ml of MEM. The PKX suspension was kept on ice until used for injection. The parasite suspension contained 1.3×10^3 PKX/ml as determined by hemocytometer counts.

Sampling procedure. All efforts were made to reduce culture-related stress. Ten fish per group were sampled from the circular tanks every week for determination of blood and serum parameters. Fish were lured to the surface with trout pellets and netted individually. The fish were quickly anesthetized in 65 ppm MS-222 (pH 7.0) and bled from the caudal vein by a 22 Ga needle into a 3 ml heparinized

vacutainer tube (Monoject). A heparinized microhematocrit tube was drawn from the vacutainer tube, plugged with clay and centrifuged at 10,000 rpm for 5 min in a microhematocrit centrifuge (IEC). The leukocrit was determined by measuring the length of the buffy coat with a calibrated ocular reticle using a 10 X objective and dividing this length by the total fluid length in the tube (McLeay & Gordon, 1977). Leukocrit was chosen as an indicator of acute stress and general health (Wedemeyer et al., 1983). Two 20 μl samples were taken for cell counts and hemoglobin measurements. A 1:100 dilution (20 μl blood:1.98 ml Rees-Ecker solution) was used for blood cell counts (Wedemeyer & Yasutake, 1977). Counts from two Neubauer chambers were averaged and the total cell count calculated (US Army Tech.manual 8-227-4) as followed:

$$RBC/mm^3 = \frac{(Ave.count)(100)}{0.02} \qquad WBC+T/mm^3 = \frac{(Ave.count)(100)}{0.4}$$

Where WBC+T was the total count of leukocytes and thrombocytes. Hemoglobin content was determined by the cyanmethemoglobin method (Drabkin & Austin, 1935). Twenty microliters of whole blood was mixed with 5 ml of Drabkin's solution, allowed to stand for 15 min, and centrifuged at 2000 rpm in an IEC tabletop centrifuge for 5 min. The absorbance at 540 nm was then read on a Bausch & Lomb Spectronic 20 spectrophotometer and the hemoglobin content (g/dL) determined from a standard curve.

A blood smear made from each fish was fixed in methanol and stained with Leishman-Giemsa (Wedemeyer & Yasutake, 1977). Leukocyte + thrombocyte counts were performed by reading at least five fields at 100 X. The number of thrombocytes, lymphocytes, granulocytes and monocytes out of 100 cells was used to determine the percentage of each cell type in circulation.

The remaining blood was centrifuged at 3,000 rpm for 10 min in a tabletop centrifuge (IEC). The plasma was stored at - 80 C. The fish's fork length (L,cm) and weight (W, g) were recorded. These values were used for calculating the condition factor ($K = 100 \times W / L^3$) (Pickering & Duston, 1983). In addition, tissue samples from the posterior kidney, gill, liver and spleen were fixed for histology. In order to determine if other pathogens were present in the trout, 10 fish from both groups were examined for bacterial and viral pathogens 5 wk into the study. Kidney material was streaked onto Brain Heart Infusion (BHI) agar plates. The plates were incubated at 25 C for 72 h and observed for colonies. Portions of the kidney and spleen from two fish were pooled and processed for virus detection by standard methods (Amos, 1985). The samples were placed onto EPC cell cultures maintained at 15 C. The cultures were observed for evidence of cytopathic effects (CPE) for 12 d.

Serological assays. During weeks 1 and 2 serum chloride, calcium, albumin, glucose, and total protein were determined

as described in the Survivor resistance: Experimental infection study. Later serological work was conducted on a DECOS CHEMISTRY ANALYZER (Coulter Electronics Corp.). The analytes determined were total protein, calcium, chloride, BUN, magnesium and glucose. Cortisol concentration in the plasma was determined from frozen (- 80 C) aliqouts by an ELISA (enzyme linked immunosorbant assay) technique (Munro & Stabenfeldt, 1985).

Plasma protein fractions were separated by electrophoresis on a cellulose acetate membrane (Beckman Microzone system). The electrophoretic cell was filled with a 0.05 M barbital buffer of pH 8.6 (Corning Universal PHAB buffer). A 0.5 μl sample of plasma was applied to a buffer-equilibrated membrane and electrophoresed at 250 V constant current for 25 min. The membranes were stained with Ponceau S protein dye, differentiated in 5% acetic acid, cleared in 23 % acid ethanol and dried for 15 min at 100 C. The relative concentration (g/dL) of each fraction was calculated from the predetermined total protein value and the integral for each fraction as determined with a Beckman scanning densitometer. The albumin/globulin ratio (A/G) was determined by dividing the albumin "F1a" fraction by all the non-albumin fractions (Ravel, 1973).

Chemiluminescence. The phagocytic activity of separated pronephros cells was evaluated by their chemiluminescent (CL) response to opsonized zymogen. One infected and one

control fish were selected for each trial. One or two trials were performed biweekly. A modified method of Hietala and Ardans (1987) was followed for the CL assay. A portion of the anterior kidney was teased through a wire screen into cold anticoagulate (HBSS-APO) consisting of 0.8 % (w/v) potassium oxalate and 1.2 % (w/v) ammonium oxalate in Hanks buffered salt solution without Ca^{2+}, Mg^{2+} or phenol red (HBSS w/o CMP). The cell suspension was filtered through a small amount of glass wool to remove bits of tissue. Four milliliters of this suspension was layered over a gradient made with 2 ml each of a 30 % and 60 % percoll in a 15 ml conical centrifuge tube and centrifuged at 300 x G for 25 min at 4 C. The percoll stock was a 9:1 percoll (Pharmacia) to 10 X PBS solution. This stock was diluted with HBSS w/o CMP to 30 % and 60 % prior to use. A leukocyte band, formed between the 30 % and 60 % bands, was removed with a pasteur pipette, washed twice in PBS (300 x G, 4 C, 15 min) and resuspended in 300 μl of HBSS w/o CMP. The cell number was determined using a hemocytometer and then adjusted with HBSS w/o CMP to reach 0.5 - 1 x 10^5 cells/mm^3. Luminol (Sigma) was used to amplify the CL reaction and serum-opsonized zymosan (Sigma) acted as the stimulus. The CL response of isolated leukocytes was monitored at 430 nm in a DuPont Luminescence Photometer (model 1760) and recorded as mV potential (range of 100) on a strip recorder (preparation of these reagents is described in Appendix 1). All solutions

were warmed to room temperatures 3 - 5 min prior to the assay. One hundred µl of the cell suspension was mixed in a reaction cuvette with 10 µl of 1:100 luminol in HBSS w/o phenol red and placed in the luminometer. After 1 min to establish a background CL reading, 30 µl of zymosan was added via a Hamilton syringe to the cuvette through an injection port. The initial slope, peak height, and time to peak were determined. In order to standardize the data for analysis, a "reaction index" (RI) was calculated (RI = Peak mV / Time to peak min). The posterior kidney was fixed for histological examination.

Plaque forming cell assay. The effect of PKD on the immune response to injected sheep red blood cells (SRBC) was examined using the plaque-forming cell (PFC) assay method of Blazer et al. (1984). Briefly, trout anesthetized with 50 ppm of MS-222 were injected I.P. with 0.1 ml of 10 % SRBC in 0.1 M PBS and held at 16 C for 14 d prior to sacrifice. The pronephros and spleen were removed and teased through a wire screen into 5 ml cold Leibovitz L-15 media supplemented with 7 % complement-inactivated (20 min at 42 C) trout serum (NTS). Clumps of tissue were removed by filtering the suspension through a small piece of glass wool. The suspension was centrifuged at 80 x G for 10 min and the cell pellet gently resuspended in 1 ml L-15 + NTS. The cell number and viability was determined using trypan blue (0.4 %) exclusion. One hundred µl of the cell suspension was

added to 15 µl pooled trout serum dilute 1:100 with PBS (complement source) and 30 µl of a 10 % SRBC suspension. Ten to twenty microliters of this mixture were introduced into plaque chambers on 25 x 75 mm microscope slides (Cunningham & Svenberg, 1968) and incubated at 25 C for 90 min. Plaques were counted and PFC/10^6 leukocytes were calculated from duplicate chambers. Pilot studies had demonstrated that non-immune trout produced a negligible background PFC response and therefore these controls were excluded.

Vaccination and bacterial challenge. The effect of PKD on both bacterin vaccinated and non-vaccinated trout to V. anguillarum challenge was examined in two experiments. The first challenge was performed 36 d post injection of either parasite suspension (PKX) or MEM to fish not vaccinated with the V. anguillarum bacterin. The second challenge took place 60 d post-injection and used both sham-vaccinated and bacterin vaccinated fish. The vaccinated groups were challenged 24 d after being exposed to bacterin or sham-bacterin.

The challenges were conducted by exposing fish to V. anguillarum in 100 L of static 16 C water containing bacterial cells from a virulent 18 h culture. An isolate of V. anguillarum (VA-32) from an epizootic among Norwegian rainbow trout (supplied by Dr. T.Hastein, National Veterinary Institute, Oslo) was used for both challenge and bacterin production. All bacterial cultures were grown in

either (BHI) broth or agar. Bacterial densities of the challenge cultures were estimated by spectrophotometry (Gould, 1977). A plate count conducted immediately after challenge confirmed the concentration of bacterial cells.

A 1.5 L bacterin solution containing approximately 1 x 10^6 cells/ml was prepared by pooling five 300 ml broth cultures of V. anguillarum. Each culture was grown for 18 h at 20 C with constant mixing on a shaker at 1600 rpm. The bacteria were inactivated by slowly adding formalin over a 1 h period to reach a final concentration of 0.4% formalin. Sterility of the bacterin was tested by plating four 0.5 ml samples on BHI agar and incubating the plates at 25 C for 5 d. The bacterin was stored at 4 C until used. Seventy fish from both the infected (PKX) and control (MEM) groups were vaccinated with V. anguillarum bacterin 36 d after their injection with PKX-suspensions or MEM respectively. An additional 70 fish from each of these groups were given a sham vaccination with 0.4 % formalin-treated BHI broth (SHAM). Replicates of 35 fish were exposed for 15 min to either 200 ml of the bacterin or SHAM solution in 100 L of 16 C water. After 15 min, water flow was resumed.

In the first V. anguillarum challenge, two replicates of 25 fish from the PKX and MEM groups were challenged with 3 x 10^4 cells/ml. The vaccinated and sham-vaccinated fish of the second challenge were segregated into two replicates of 25 fish each and challenged with 2 x 10^4 cell/ml. In

addition, 20 fish from both the bacterin and sham groups not challenged served as negative controls.

Mortalities occurring over a 12 d period from each challenge were collected and examined by streaking kidney tissue onto BHI agar plates. After 24 h at 25 C, the plates were examined for the presence of V. anguillarum colonies. Gross lesions of the fish, colony morphology, cell motility, Gram reaction, and novobiocin sensitivity were used to confirm the presence of V. anguillarum. Kidney samples were taken from PKX-infected survivors of the challenges for histological examination.

Physiological & Immunological Parameters:

Field exposure

This study examined the effect of PKD on blood and immune parameters of trout infected by natural exposures (estimated 3 - 6 wk) to the water-borne infectious stage and later held under laboratory conditions. The results of this study were compared to those generated in the experimental infection study in which a single dose of parasites was used to infect the host.

Study groups. Eight hundred and fifty 0+ rainbow trout (AR-RTH-86) were transported from ARH to the UCD-FDL on March 27, 1987. These control fish had an average weight of

10.1 g (SD 4.1) and were designated AR-CON. The controls were maintained at 15 C in 650 L circular tanks. These underyearling fish were considered to be naive to PKX as they had been moved to ARH in the winter and histological examination of 15 trout on May 14, 1987 revealed no PKX cells in the kidney. Biweekly sampling of the same population of trout (RTH) remaining at ARH detected the first PKX infections on May 27. In this sample, 12 out of 20 fish had a low parasitemia observed in kidney vessels. Fourteen days later on June 12, 800 AR-RTH fish (avg wt 40.5 g) were transferred to the UCD-FDL. These fish were designated AR-PKD. The fish were prophylactically treated enroute (1 h) with 10 ppm nitrofurazone (Argent Laboratories) in order to prevent Flexibacter columnaris infections. On the same day, the AR-CON group was also treated with nitrofurazone and transferred to another circular tank.

Sampling procedure. A sampling protocol similar to that used in the experimental infection study was followed with three exceptions. First, 20 fish from each group were sampled biweekly for physiological parameters instead of 10 fish/group each week, blood cell counts were not done, and spleen samples were fixed in 10 % buffered formalin and the paraffin sections stained by the Prussian blue method for iron deposits (Humason, 1979). The serological parameters examined and their assay methods were identical to the

experimental infection study. On July 15, 1987, 20 fish/group were assayed for bacterial pathogens and on August 11 the same number of fish were sampled for virus isolation. Methodology was the same as in the experimental infection study.

Plaque forming assay. The agarose slide method of Anderson et al. (1979a) was used to examine the effect of PKD on the immune response to injected sheep red blood cells (SRBC). The selection of this adaptation of the Jerne agar slide method over the PFC method used previously in the experimental infection study (Blazer et al., 1984) was based on two factors. First, there were problems with volume regulation (used to determine the number of cells per chamber) when filling the PFC slide chambers. Also, Blazer et al. (1984) method was much more time consuming than the agar slide method.

Fish were anesthetized with MS-222, injected with 0.2 ml IP of a 10 % SRBC suspension in PBS, and maintained at 15 - 16 C . After 14 d, the fish were sacrificed and a leukocyte suspension from the anterior kidney and spleen of each fish was prepared as previously described. The posterior kidney from each fish was fixed in Davidson's solution. One hundred microliters of cell suspension was added to 200 μl of 12 % SRBC and plaquing media (16 C). The plaquing medium was prepared by adding 100 μl of boiled 1 % agarose (low temperature type, Sigma A-4018) in distilled

water to 50 µl of 2 X MEM. Both the 2 X MEM and the 1 % agarose were kept in a 16 C water bath prior to mixing. The PFC suspension was mixed then poured onto a 25 x 75 mm slide which had been pre-coated with 0.1 % agarose. The slide solidified as it cooled on a level surface for 10 min. It was placed in a 25 C moist chamber for 4 h. A complement source of normal trout serum diluted 1:8 with PBS was then pipetted onto the slide. The slide was examined for plaques 4 h later. Duplicate PFC slides were prepared from each fish. Three 14 d PFC trials were completed with the AR-PKD and AR-CON fish on July 22, July 29, and August 15, 1987.

Nitroblue tetrazolium assay. The oxidative metabolism of stimulated pronephros cells was measured in a nitroblue tetrazolium (NBT) assay (Alfoldy & Lemmel, 1979). A modification of the method of Bachner & Nathan (1968) was employed for the assay. Pronephros cells were prepared as described for the PFC assay and their number adjusted to 2.0 - 2.8 x 10^5 cells/mm^3. One hundred and fifty microliters of the cell suspension was added to 50 µl of serum-opsonized zymosan (Sigma) and 100 µl of 0.5 % (w/v) KCN in saline. The mixture was gently agitated for 10 min at room temperature and 100 µl of 2 mg/µl NBT (Sigma) in saline was then added. After an additional incubation period of 30 min, the reaction was stopped by adding 0.5 N HCl. The mixture was centrifuged at 1100 rpm for 5 min in an IEC tabletop

centrifuge. The supernatant was removed and 4 ml of pyridine was pipetted onto the cell pellet. The pyridine mixture was capped and placed in 100 C water bath for 10 min. Three ml of the supernatant was examined for absorbance in a Spectronic-20 spectrophotometer (Bausch & Lomb) at 515 nm. Duplicate tubes were prepared from the pronephros cells of each fish. A total of three NBT trials were performed on July 16, 22 and July 28, 1987. Posterior kidneys were saved for histological examination.

Vaccination and bacterial challenge. The bacterial culture, bacterin production, mortality screening, and challenge procedures were identical to those in the experimental infection study. Three challenges were conducted during the study. The first challenge on June 16, 1987 consisted of two 25 fish replicates from both the AR-CON and AR-PKD groups. These non-vaccinated fish were challenged with 3×10^4 cells/ml and held at 15 C for 12 d. Twenty days later (July 6, 1987) fish vaccinated on June 16 with either the V. anguillarum bacterin or sham bacterin were challenged with 1.2×10^6 cells/ml. The water temperature was maintained at 16 C for the 12 d period. On August 9, 1987 (59 d after transport of the AR-PKD fish to the lab), another lot of fish vaccinated 20 d earlier were challenged with 6×10^4 cells/ml and the average water temperature for the 12 d period was 15.1 C. In both the July 6 and August 9 challenges, bacterin and sham-vaccinate

fish were divided into two 28 fish replicates and challenged with V. anguillarum. In addition, 20 fish from the bacterin and sham groups were not challenged and served as negative controls. Kidney samples for later examination were taken from fish in the AR-PKD group that survived challenge.

Statistics

Chi - squared tests were used for comparisons of mortality in groups of fish. Student's T - test was used to compare the significance of the data obtained in all other assays. A significance level of $P<0.05$ was chosen for all tests.

RESULTS

Survivor resistance to field exposure.

The sentinel fish study revealed that the infectious stage of PKD was present in ARH water supply during September and October 1984 (Table 2). PKX organisms were not detected in kidney sections of either the ambient or heated water groups exposed from November 1984 through March 1985. No further infections occurred until fish were exposed at ARH in April 1985. The infectious stage was then present through September 1985. The April exposure groups experienced acute mortalities in the laboratory due to gas supersaturation and the sample size was reduced.

Ambient water temperatures ranged from 12 to 20 C during the time the infectious stage was present. The prevalence of infection among the sentinel fish peaked at 97 % in the June 1985 exposure group. The greatest degree of interstitial hyperplasia corresponded with large numbers of interstitial parasites. The high prevalence of infection (88 %) in the May 1985 exposure group prompted the June 1985 start of the survivor resistance study.

The production lots, Group B and C, closely matched the sentinel fish in their observed disease cycle. PKX infection in both groups was first detected in June 1985. Group B had been introduced to ARH in October 1984, a time when the sentinel fishes were demonstrating that the infectious stage was present in the water supply (Fig. 5). Therefore, this

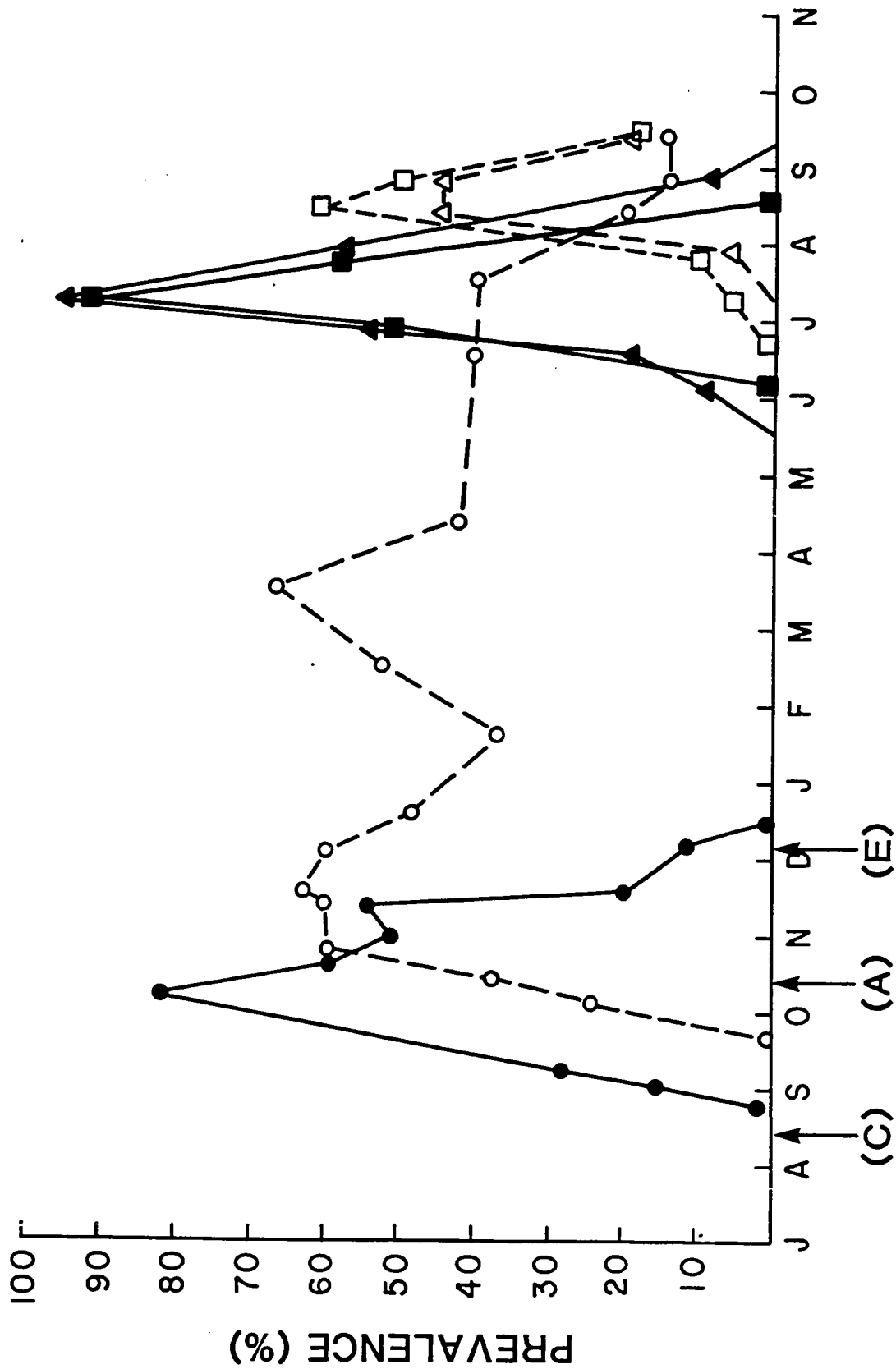
Table 2. Prevalence of the parasite PKX in groups of rainbow trout exposed each month to the water supply of the American River hatchery.

Exposure Initiation	INTERSTITIAL PKX No. infected (%) / No. examined: Ambient	INTERSTITIAL PKX: 17 C*	INTRALUMINAL PKX No. infected (%) / No. examined: Ambient	INTRALUMINAL PKX: 17 C	Mean Water Temp.(C)
09-06-84	4/19 (21)	ND	7/19 (37)	ND	19
10-10-84	2/6 (33)	ND	1/6 (17)	ND	17
11-12-84	0/7 (0)	0/18 (0)	0/7 (0)	0/18 (0)	13
12-12-84	0/18 (0)	ND	0/18 (0)	ND	11
01-13-85	0/20 (0)	0/16 (0)	0/20 (0)	0/16 (0)	7
02-20-85	0/21 (0)	0/12 (0)	0/21 (0)	0/12 (0)	7
03-18-85	0/31 (0)	0/27 (0)	0/31 (0)	0/27 (0)	10
04-17-85	7/8 (88)	2/6 (33)	0/8 (0)	0/6 (0)	12
05-15-85	25/31 (81)	23/25 (92)	2/31 (6)	0/25 (0)	13
06-15-85	31/32 (97)	ND	12/32 (38)	ND	15
07-16-85	22/30 (73)	ND	8/30 (27)	ND	17
08-13-85	1/10 (10)	ND	0/10 (0)	ND	20
09-10-85	1/23 (4)	ND	0/23 (0)	ND	19
10-15-85	0/31 (0)	ND	0/31 (0)	ND	17

* During the period when ambient water temperature fell below 15 C, a second sentinel group was exposed to hatchery water heated to 17 C.
ND Not done.

Fig. 5

Prevalence of infection of PKX interstitial (——) and intraluminal (---) stages in the kidneys of three groups of rainbow trout at American River hatchery, A (●, ○), B (▲, △), and C (■, □). Dates of introduction to the hatchery are noted by arrows.



group was considered to have been exposed to the parasite prior to spring 1985. Group C was introduced to ARH in December 1984 when the sentinel fish remained uninfected, and fish in Group C were considered naive to the parasite prior to the spring outbreak. A peak prevalence of infection of 91% (Group B) and 95% (Group C) occurred in July (Fig. 5). The interstitial stages of PKX could no longer be detected in fish from either group by September 1985. Monthly mortality in these fish ranged from 0.04 % to 6.0 % during the summer, however, infections with F. columnaris were also occurring at this time.

Infection with the PKX parasite was first detected in Group A 3 wk after their introduction to ARH in September 1984 (Fig.5). The prevalence of infection for the interstitial PKX stage peaked 4 wk later. Interstitial PKX parasites were not detected in kidney sections beginning in December 1984, and neither clinical signs of PKD nor interstitial PKX stages were observed in the fish during the 1985 summer epizootic. Sporogonic stages were first observed in the lumina of kidney tubules 7 wk after the introduction of the Group A fish and could be detected for 13 mo. Both the prevalence of infection and the intensity (number of parasites per kidney) of luminal stages declined from a peak in March 1985.

No interstitial PKX parasites or kidney lesions typical of PKD was observed in the Group A yearling fish used in the

survivor resistance study. When sampled at 8 wk post-exposure, the naive control trout had a prevalence of infection for interstitial and intraluminal stages of 96 % (44/46) and 17% (8/46), respectively. These naive yearlings had lesions typical of PKD with 70 % having histological lesions that were given a moderate or heavy PKD rating.

Survivor resistance: experimental infection.

Interstitial PKX parasites were not observed in any kidney sections from either the MEM or PKX injected survivor fish (AR). Eight weeks after injection, the prevalence of infection for intraluminal stages was 12 % and 14 % for the PKX and MEM injected survivor fish, respectively. In contrast, the PKX injected naive trout (DS-PKX) had 89 % (25/28) prevalence of interstitial PKX infection with 61 % (17/28) having a moderate to heavy PKD ratings (Table 3). Histologically, renal lesions were focal with discrete granulomas scattered throughout the kidney. Intraluminal forms were seen in 29 % (8/28) of the DS-PKX kidney sections. Kidneys of the control, naive trout (DS-MEM) did not contain interstitial or intraluminal parasites. Trematodes were seen in 13 % (7/56) of all DS kidney sections. Darrah Springs hatchery has a history of Sanquincola davsi infections (Wales, 1958).

The mean (SD) fork length and weight for the two groups were: Survivor AR-ELT fl = 22.44 cm (2.32) and wt = 138.37 g (40.98); Control DS-RTS/RTKJ fl = 23.00 cm (2.64) and

Table 3. Prevalence of infection in proliferative kidney disease survivor (AR) and control (DS) rainbow trout experimentally infected with the PKX parasite by intraperitoneal injection.

	No. positive / No. examined (%)		
GROUP*	PKX	INTRALUMINAL	M / H LESION**
DS-PKX (61)	25/28 (89)	8/28 (29)	17/28
DS-MEM	0/26 (0)	0/26 (0)	0/26 (0)
AR-PKX	0/26 (0)	3/26 (12)	0/26 (0)
AR-MEM	0/28 (0)	4/28 (14)	0/26 (0)

* Fish were injected intraperitoneally with 0.2 ml of a kidney suspension containing the parasite (PKX) or minimum essential media (MEM). Fish were sacrificed for histological examination at 8 wk post-injection. The average weights for the control trout (DS) and PKD survivor trout (AR) at the conclusion of the study were 138.4 g and 147.6 g respectively.

** Infected fish were classified into three groups (L = light, M = moderate, or H = heavy) by the severity of microscopic lesions of the kidney.

wt = 147.56 g (53.67). The yearling fish used in this experiment were similar in size and age, however, several strain differences were observed. There were significant differences ($P < 0.05$) between the leukocrit and albumin values from MEM injected fish of the two strains (Table 4, Appendix 2 & 3). It was decided from these data to limit comparisons between treatment groups of the same strain. While the mean hematocrit of the DS-PKX group was statistically less than the DS-MEM controls, anemia was not apparent (Table 4). Leukocrits did not vary between treatment groups and the relative percent of leukocytes and thrombocytes remained similar (Table 5). No major changes were seen in the calcium, total protein, albumin, or chloride values between the PKX and MEM treatment groups (Appendix 2 & 3).

Effect of serum from survivor trout on parasite infectivity.

All three groups injected with PKX suspensions (incubated with either saline, nonimmune or immune sera) had high prevalences of infection (89 - 91 %) at 7 wk post-injection regardless of the type of treatment given to the suspension (Table 6). The prevalence of fish with moderate to heavy disease ratings were also high (63 - 76 %) in these groups. Parasites with daughter cells were seen in all infected fish (Fig. 6). Intraluminal stages were observed in only a few fish (Fig. 7). The fish injected only with saline (negative controls) remained uninfected.

Table 4. Hematocrit and leukocrit values of experimentally infected proliferative kidney disease (AR) and control (DS) rainbow trout.

GROUPS*	HEMATOCRIT MEAN	HEMATOCRIT STD.	LEUKOCRIT MEAN	LEUKOCRIT STD.
DS-PKX n = 28	35.9+	5.2	1.264	0.716
DS-MEM n = 26	42.1	4.7	1.293	0.311
AR-PKX n = 26	36.2	9.6	0.853	0.389
AR-MEM n = 28	40.3	8.0	0.953	0.457

* Fish were injected intraperitoneally with 0.2 ml of a kidney suspension containing the parasite (PKX) or minimum essential media (MEM). Fish were sacrificed for histological examination at 8 wk post-injection. The average weights for the control trout (DS) and PKD survivor trout (AR) at the conclusion of the study were 138.4 g and 147.6 g respectively.

\+ Significant at $P < 0.05$. Comparisons between treatment groups within the same stock.

Table 5. Relative percent of lymphocytes, thrombocytes, monocytes and granulocytes counted from 100 non-erythrocyte cells per bloodsmear of infected PKD survivor (AR) and control (DS) rainbow trout*.

GROUP	LYMPHOCYTE	THROMBOCYTE	MONOCYTE	GRANULOCYTE
AR-MEM				
Mean	77.4	20.1	0.4	2.1
SD	10.8	9.8	0.3	2.0
n = 12				
AR-PKX				
Mean	68.6	25.2	C.7	4.7
SD	13.1	10.4	0.8	4.8
n = 10				
DS-MEM				
Mean	74.3	21.3	0.5	1.8
SD	12.9	12.3	0.6	1.7
n = 12				
DS-PKX				
Mean	76.8	19.1	2.6	1.4
SD	13.3	14.1	2.1	1.2
n = 12				

* Fish were injected intraperitoneally with 0.2 ml of a kidney suspension containing the parasite (PKX) or minimum essential media (MEM). Fish were sacrificed for histological examination at 8 wk post-injection. The average weights for the control trout (DS) and PKD survivor trout (AR) at the conclusion of the study were 138.4 g and 147.6 g respectively.

Figure 6-8. Interstitial and intraluminal stages of the PKX parasite in the kidneys of rainbow trout, O. mykiss. P = Primary cell, D = daughter cells, M = macrophage. Bar = 10 μm. H & E. 6 μm sections. 6. Multicellular PKX parasites in kidney interstitium. 7. PKX parasites in renal vessels. 8. Intraluminal stages.

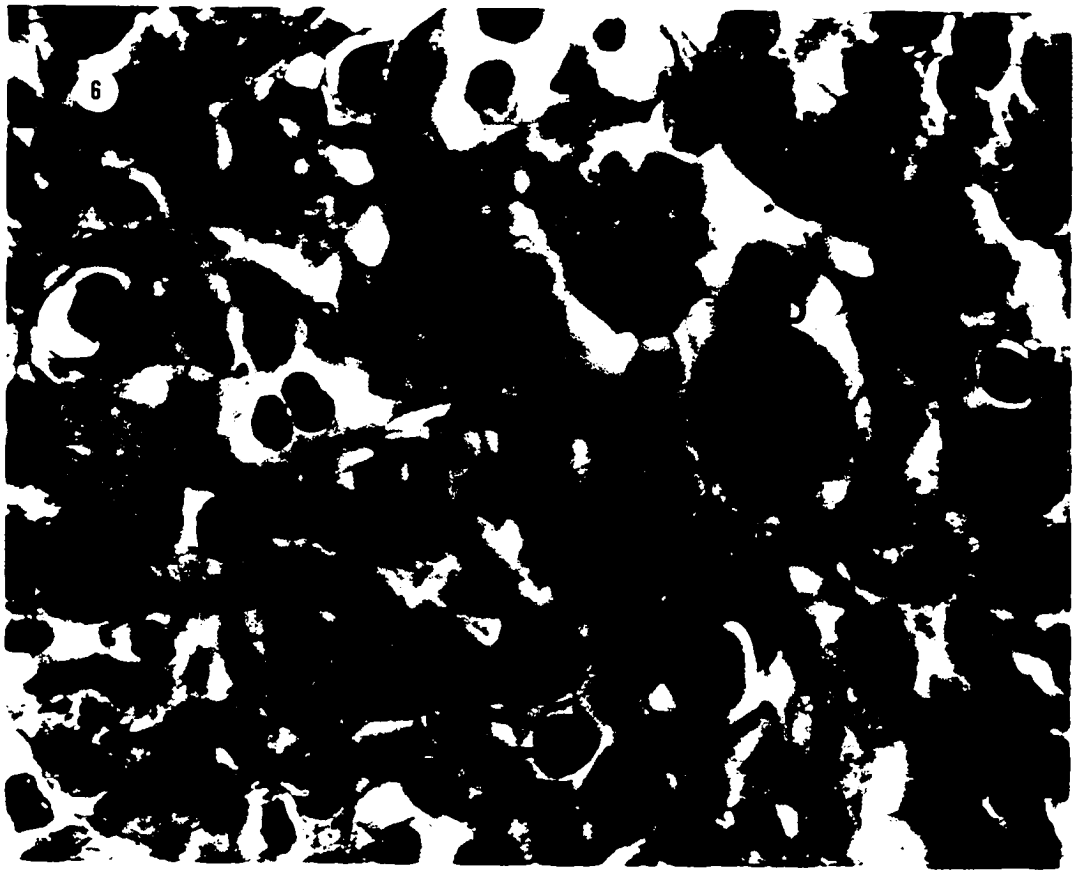


Table 6. Effect of serum from rainbow trout which had recovered from proliferative kidney disease on the experimental infectivity of the PKX parasite.

	No. positive / No. examined (%)		
GROUP*	PKX	INTRALUMINAL	M or H LESION**
PKX-NS n = 35	30/35 (86)	2/35 (6)	22/35 (63)
PKX-IS n = 33	29/33 (88)	3/33 (10)	24/33 (73)
PKX-SAL n = 33	30/33 (91)	3/33 (10)	25/33 (76)
SALINE n = 13	0/13 (0)	0/13 (0)	0/13 (0)

* A PKX-infected kidney suspension was incubated for 30 min at 25 C in either a 1:2 mixture of immune survivor trout serum (IS),nonimmune control trout serum (NS),or saline (SAL) and injected intraperitoneally into rainbow trout (avg wt 23.1 g). Another group was injected with saline only. Kidney sections of the IS donors did not contain interstitial PKX.

** Fish were categorized into four groups (None, L = Light, M = Moderate, or H = Heavy) by the degree of microscopic lesions of the kidney.

Physiological & Immunological Parameters:

Experimental Infection

Disease course. Interstitial PKX parasites were first observed in the kidney sinuses of two fish sampled 23 d post-injection (Fig. 8). The prevalence of infection (90 %) and the number of fish with moderate - to - heavy disease (60 %) peaked at 43 d post-injection (Table 7). A recovery phase followed in which both parasite number and renal lesions diminished. Intraluminal forms were observed in only two fish during the last 3 wk of the study. Focal regions of interstitial hyperplasia characterized the microscopic lesions observed during the study. Few fish had grossly swollen kidneys. Eggs and miracidia of the trematode Sanguinicola davisi were observed in approximately 60 % of gill sections from these Darrah Springs hatchery fish. Diffuse gill hyperplasia and large numbers of parasites were rarely seen. There was no correlation between the number of miracidia in the gill lamellae and the degree of PKD. Cross-sections of adult flukes were seen in only eight kidney sections from the study. Pansporoblasts of myxosporean Chloromyxum sp. were also seen in glomeruli of seven fish, however, no lesions were associated with the pansporoblasts. Condition factor was not affected by PKX infection (Table 8). The mean condition factors of the PKD-L and PKD-M fish were statistically greater than that of the

Table 7. Prevalence of parasite infection and severity of lesions in rainbow trout (DS-RTS) experimentally infected with proliferative kidney disease.

DAYS POST PKX INJECTION	No. positive / No. examined (%) PKX	M or H LESION+	INTRALUMINAL
8	0/10 (0)*	0/10 (0)	0/10 (0)
17	0/10 (0)	0/10 (0)	0/10 (0)
23	2/10 (20)	0/10 (0)	0/10 (0)
29	2/10 (20)	0/10 (0)	0/10 (0)
36	5/10 (50)	2/10 (20)	0/10 (0)
43	9/10 (90)	6/10 (60)	0/10 (0)
51	5/10 (50)	4/10 (40)	1/10 (10)
58	6/10 (60)	2/10 (20)	0/10 (0)
69	3/10 (30)	1/10 (10)	1/10 (10)

+ Infected fish were classified into three groups (L = light, M = moderate, or H = heavy) by the severity of microscopic lesions of the kidney.

Table 8. Effect of experimental proliferative kidney disease on fork length (FL), weight (W), and condition factor (K-FACT) of rainbow trout (DS-RTS).

GROUP[+]	FL (cm)	W (g)	K-FACT[++]
MEM			
Mean	24.3	188.1	1.269
SD	2.3	59.6	0.099
n = 60			
PKD-L			
Mean	23.9	184.4	1.312*
SD	2.4	62.2	0.117
n = 18			
PKD-M			
Mean	26.0	254.1	1.428**
SD	1.6	57.8	0.103
n = 7			
PKD-H			
Mean	22.9	154.2	1.271
SD	0.7	18.7	0.079
n = 7			

\+ Fish were injected intraperitoneally with 0.2 ml of infected kidney suspension (PKD) or minimum essential media (MEM).Infected fish were classified into three groups (L= light, M = moderate, or H = heavy) by the degree of microscopic pathology of the kidney.

++ Condition factor= $100^* W/L^3$.

* Significantly different from MEM controls at $P < 0.05$.

** Significantly different from MEM controls at $P < 0.01$.

control group. No bacterial or viral agents were isolated from fish in either the PKD or MEM groups.

Hematology. Signs of anemia were not observed in infected fish, however, the mean hematocrit (Hct) and erythrocyte counts (RBC) tended to be lower than those of the controls (Table 9). While the Hct of the PKD-M fish and the RBC count of the PKD-L and PKD-H fish were lower than those of the MEM controls, these values were not statistically significant ($0.05 < P < 0.10$). Hemoglobin concentrations were similar for all groups. Of the erythrocyte indices, only the mean corpuscular volume (MCV) of the PKD-L group was significantly different from that of the control group (Table 10). The mean leukocrit values of the infected fish were above that of the controls, with the PKD-H fish significant at $P < 0.05$ (Table 9). Leukocyte and thrombocyte (WBC-T) hemocytometer counts did not follow the leukocrit trend as no significant differences were observed (Table 9). Leukocyte and thrombocyte counts from blood smears did not reveal any differences between the groups in the composition of non-erythrocyte cells of the blood (Table 11).

Serology. The infected groups had decreased plasma chloride concentrations compared to the controls, however, the differences were not significant ($0.05 < P < 0.10$) (Table 12). There was no apparent correlation in chloride

Table 9. Effect of experimental proliferative kidney disease on hematocrit (Hct), leukocrit (Lct), hemoglobin concentration (Hb), erythrocyte (RBC) and white blood cell and thrombocyte count (WBC-T) in rainbow trout (DS-RTS).

GROUP[+]	Hct	Lct	Hb (g/dl)	RBC (10^6/mm^3)	WBC-T (10^4/mm^3)
MEM					
Mean	35.5	1.0482	8.1	1.068	6.764
SD	4.5	0.2287	1.4	0.142	2.170
n = 60					
PKD-L					
Mean	34.3	1.1379	7.6	0.953	6.594
SD	3.5	0.2798	1.0	0.182	1.851
n = 18					
PKD-M					
Mean	33.0	1.1862	7.8	1.000	7.748
SD	2.6	0.1934	0.9	0.161	2.272
n = 7					
PKD-H					
Mean	35.1	1.2732*	7.5	0.975	6.291
SD	4.1	0.2259	0.5	0.085	2.077
n = 7					

+ Fish were injected intraperitoneally with 0.2 ml of infected kidney suspension (PKD) or minimum essential media (MEM). Infected fish were classified into three groups (L = light, M = moderate, or H = heavy) by the severity of microscopic lesions of the kidney.

* Significantly different from MEM controls at $P < 0.05$.

Table 10. Effect of experimental proliferative kidney disease on mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) in rainbow trout (DS-RTS).

GROUP+	MCV (μm^3)	MCH (10^{-12} g)	MCHC
MEM			
Mean	339.3	76.9	22.9
SD	76.0	16.1	3.5
n = 60			
PKD-L			
Mean	378.3*	83.5	22.1
SD	99.5	24.3	2.5
n = 18			
PKD-M			
Mean	343.7	79.1	23.4
SD	63.7	8.3	2.6
n = 7			
PKD-H			
Mean	372.8	77.5	21.0
SD	68.7	11.5	2.0
n = 7			

+ Fish were injected intraperitoneally with 0.2 ml of infected kidney suspension (PKD) or minimum essential media (MEM). Infected fish were classified into three groups (L = light, M = moderate, or H = heavy) by the severity of microscopic lesions of the kidney.

* Significantly different from MEM controls at $P < 0.05$.

Table 11. Effect of experimental proliferative kidney disease on relative percent of lymphocytes, thrombocytes, monocytes and granulocytes counted from 100 non-erythrocyte cells per bloodsmear of rainbow trout (DS-RTS).

GROUP+	THROMBOCYTE	GRANULOCYTE	LYMPHOCYTE	MONOCYTE
MEM				
Mean	9.6	2.5	87.9	0
SD	6.9	2.6	9.5	0
n = 30				
PKD-L				
Mean	9.4	2.4	87.2	0
SD	5.9	2.7	5.1	0
n = 10				
PKD-M				
Mean	12.5	2.3	85.2	0
SD	13.7	2.9	16.4	0
n = 6				
PKD-H				
Mean	9.5	2.3	87.8	0
SD	11.3	2.9	10.9	0
n = 8				

+ Fish were injected intraperitoneally with 0.2 ml of infected kidney suspension (PKD) or minimum essential media (MEM). Infected fish were classified into groups (L = light, M = moderate, or H = heavy by the severity of microscopic lesions of the kidney.

Table 12. Effect of experimental proliferative kidney disease on plasma chloride, calcium, glucose, magnesium and total protein concentrations of rainbow trout (DS-RTS).

GROUP*	CHLORIDE mmol/l	CALCIUM mg/dl	GLUCOSE mg/dl	TOTAL PROTEIN g/dl	MAGNESIUM mg/dl
MEM					
Mean	132.36	11.23	82.29	3.63	2.03
SD	4.88	1.07	8.77	0.15	0.22
n = 30					
PKD-L					
Mean	128.33+	10.85	75.83+	3.72	2.15
SD	3.50	0.55	6.39	0.25	0.15
n = 8					
PKD-M					
Mean	128.40+	10.78	87.40	3.92++	2.23+
SD	1.96	0.27	15.93	0.16	0.13
n = 6					
PKD-H					
Mean	128.14+	10.89	86.71	3.87++	2.19+
SD	2.47	0.29	11.31	0.18	0.22
n = 7					

* Fish were injected intraperitoneally with 0.2 ml of a kidney suspension containing the parasite (PKD) or minimum essential media (MEM). Infected fish were classified into three groups (L = light, M = moderate, or H = heavy) by the severity of microscopic lesions of the kidney.

\+ Significantly different from MEM controls at $P < 0.05$.

++ Significantly different from MEM controls at $P < 0.01$.

concentration and the degree of renal involvement as all three groups of infected fish had similar mean values. Plasma calcium values did not change due to infection, however, magnesium concentrations increased in the infected groups (PKD-M and PKD-H groups, $P < 0.05$). All glucose values were within the normal range for yearling rainbow trout (McCarthy et al., 1975), however, the mean glucose value for the PKD-L was significantly ($P < 0.05$) less than that of the MEM controls. Plasma cortisol means in μg/dl (SD) for the groups were as follows: MEM 2.06 (1.58) n = 14, PKD-H & M 1.94 (0.82) n = 7, PKD-L 1.85 (1.11) n = 6. The mean plasma cortisol values did not differ from each other at $P < 0.05$. Plasma protein levels of the PKD-M and PKD-H fish were significantly higher ($P < 0.01$) than those of the controls. Five plasma protein fractions were consistently separated by electrophoresis (Table 13). There was a marked change in the plasma electrophoretic pattern of the PKD-M and PKD-H fish. The pre-albumin/albumin fraction (F1a) and a globulin fraction (F3) declined while globulin fractions (F4) and (F5) increased. This protein distribution resulted in a significantly lower A/G ratio than that of the control group.

Chemiluminescent assay. No relationship between PKD and the CL response was observed in the DS trout. PKX-infected fish, regardless of their disease rating, demonstrated

Table 13. Effect of proliferative kidney disease on the protein distribution* of rainbow trout (DS-RTS)** plasma separated by electrophoresis.

Fraction	MEM (n = 15)	PKD-L (n = 6)	PKD-M (n = 6)	PKD-H (n = 7)
F1a	53 (6.6)	46 (0.7)	43 (4.3)+	42 (3.4)+
F2	11 (2.2)	12 (0.4)	12 (0.5)	11 (0.1)
F3	19 (3.9)	19 (0.2)	15 (0.3)+	17 (1.8)+
F4	11 (1.5)	15 (0.1)+	15 (0.1)+	13 (0.1)+
F5	6 (0.8)	8 (0.3)	15 (0.3)+	17 (3.4)+
A/G++	1.18 (.31)	0.87 (.26)	0.61 (.17)+	0.75 (.10)+

* Cellulose acetate membrane electrophoresis at 250 V for 25 min. Total protein value and densitometer scanning pattern used to calculate the relative percentage for each fraction.

** Fish were injected intraperitoneally with 0.2 ml of a kidney suspension containing the parasite (PKD) or minimum essential media (MEM). Infected fish were classified into three groups (L = light, M = moderate, or H = heavy) by the severity of microscopic lesions of the kidney.

\+ Significantly different from MEM controls at $P < 0.01$.

++ Albumin/globulin ratio.

response indices (RI) that were both lower (3 out of 5 cases) and higher (2 of 5) than their respective MEM controls (Table 14). The average R.I. values (SD) for the groups were as follows: PKX = 12.89 (7.45), n = 5 ; MEM = 11.16 (9.19) , n = 12. Average R.I. values were not significant at $P < 0.05$. There was a large individual variability in CL responses. The infected fish generally had a 2 - 3 X greater concentration of anterior kidney cells in the initial, unadjusted suspension than their MEM controls due to renal hyperplasia.

Plaque forming cell assays. In both the 36 and 64 d post parasite injection PFC assays, the PFC responses were quite similar (not significant $P < 0.05$) between the PKX and MEM groups (Table 15). The 36 d PKX fish were near the peak of the disease cycle as determined by the severity of histological lesions and parasite numbers in their kidneys. The 64 d infected fish were in a recovery phase as characterized by lesion resolution and a paucity of interstitial PKX stages.

Vaccination and bacterial challenge. Trout infected by the PKX parasite 36 d prior to challenge by *V. anguillarum* had a significantly higher survival rate than their MEM controls (Table 16). The microscopic lesions associated with PKX infection in trout surviving *V. anguillarum* challenge showed the fish were near the peak of the disease. The prevalence

Table 14. Effect of experimental proliferative kidney disease on the chemiluminescent (CL) response of trout (DS-RTS) pronephros cells.

WEEKS POST INJECTION	FISH	PEAK mV min. to PEAK	RESPONSE+ INDEX	DEGREE OF PKD
1	P1	37/22	1.68	0
	M1	134/14	9.57	
	P2	104/13	8.00	0
	M2	25/22	1.14	
	P3	44/32	1.38	0
	M3	ND		
2	P4	90/3	30.00	0
	M4	18/7	2.57	
	P5	140/8	17.50	0
	M5	190/17	11.18	
	P6	43/5	8.60	0
	M6	82/18	4.56	
4	P7	40/28	1.43	0
	M7	60/4	15.00	
	P8	29/4	7.25	L
	M8	105/28	3.75	
	P9	97/20	4.85	0
	M9	97/34	2.85	
6	P10	146/8	18.25	H
	M10	169/7	24.15	
	P11	45/2	22.50	H
	M11	135/10	13.50	
8	P12	60/13	4.63	L
	M12	195/13	15.00	
	P13	142/12	11.83	M
	M13	184/6	30.67	

+ Reaction index = PEAK mV / min to PEAK.

Table 15. Effect of experimental proliferative kidney disease on the plaque forming cell (PFC)* response of rainbow trout (DS-RTS).

GROUP**	DAYS POST PKX INJECTION	PFC/10^6 leukocytes (SD)
MEM n = 7	36	206 (64)
PKD n = 5	36	156 (24)
MEM n = 6	64	105 (81)
PKD n = 3	64	100 (47)

* Fish were injected intraperitoneally with 0.5 ml of 10% sheep red blood cells and isolated leukocytes assayed in slide chambers for the PFC response 14 d later.

** Fish were injected intraperitoneally with 0.2 ml of a kidney suspension containing the parasite (PKD) or minimum essential media (MEM). Infection was determined by histological examination.

Table 16. Effect of experimental proliferative kidney disease at 36 d post-infection on the survival of rainbow trout* (DS-RTS) following challenge** with Vibrio anguillarum.

No. mortalities / No. exposed (%)		
PKD	MEM	
19/51 (38)	32/51 (64)	P<0.05

* Fish were injected intraperitoneally with 0.2 ml of a kidney suspension which contained the parasite (PKD) or minimum essential media (MEM). The average weight of the PKX and MEM fish were 149.0 g (SD = 23.4) and 153.8 g (SD = 10.7) respectively.

** Fish were challenged with an 18 h culture of Vibrio anguillarum. Mortalities were monitored for 12 d. The challenge dose and mean water temperature was 3.2×10^4 cells/ml and 15.3 C.

of infection and number of fish with moderate-to-heavy disease rating was 65.6 % (21/32) and 31.3 % (10/32) respectively.

The PKX-injected fish (sham and bacterin) used in the vaccination trial showed an almost identical response to V. anguillarum challenge as their respective MEM controls (Table 17). The challenge occurred 60 d following the injection of either MEM or parasites. Both groups given the sham-bacterin sustained a high mortality rate (PKD = 54 %, MEM = 56 %) in comparison to the bacterin vaccinated fish (PKX = 4 %, MEM = 2 %). Kidney tissues examined from 22 PKD-sham and 14 PKD-bacterin treated trout that survived V. anguillarum challenge showed that these fish were in the early recovery phase of the disease. The prevalence of infection (PKX) and number of fish with moderate - to - heavy disease (M/H) were as follows: (PKD-sham) PKX = 40.9 % (9/22) and M/H = 22.7 % (5/22); (PKD-bacterin) PKX = 35.7 % (5/14) and M/H = 21.4 % (3/14).

Physiological & Immunological Parameters:

Field Exposures

Disease course. The presence of a low-intensity PKX infection of the renal vasculature in the May 27 ARH sample indicated that the population had been exposed to the infectious stage 2 - 4 wk prior, and the fish were probably

Table 17. Effect of experimental proliferative kidney disease at 60 d post-infection on the response to bacterin vaccination* and challenge with Vibrio anguillarum in rainbow trout+ (DS-RTS).

	no. mortalities / no. exposed (%)	
	PKD	MEM
BACTERIN	2/50 (4)	1/50 (2)
NS		
SHAM	27/50 (54)	28/50 (56)
NS		

* Trout were vaccinated by a 15 min bacterin bath containing 10^6 cells/ml formalin-killed, V. anguillarum. The fish were challenged after 24 d at 16 C with a 15 min bath of an 18 h V. anguillarum culture of 2 x 10^4 cells/ml. Mortalities were monitored for 12 d. Sham-vaccinated groups received identical treatment except a 0.4 % formalin BHI broth was used instead of bacterin. No mortality occurred in the 40 unchallenged control fish for both PKD and MEM groups.

\+ Fish were injected intraperitoneally with 0.2 ml of a kidney suspension containing the parasite (PKD) or minimum essential media (MEM). The average weights (SD) of the PKD and MEM fish were 201.2 g (20.3) and 214.4 g (16.3) respectively.

NS Not significant at $P < 0.05$.

in the early stages of PKD when the study began (Kent & Hedrick, 1987). The peak prevalence of infection and renal lesions occurred in the second and fourth week of the study respectively (Table 18). Renal swelling was observed only in fish classified in the PKD-H group. Upon the conclusion of the study on August 11, the PKD fish were well into a recovery phase as characterized by increased prevalence of intraluminal sporogonic forms in the lumina of kidney tubules, reduction in the prevalence of interstitial PKX parasites, and resolution of lesions. The prevalence of intraluminal stages in the biweekly samples varied between 5 - 30 %. There was some variation in the disease state observed in fish within the same sample period.

Condition of Fish. The mean condition factor (K) of the PKD-M and PKD-H fish were similar to that of the control group (Table 19). No bacterial or viral pathogens were isolated from either group.

Hematology. The mean hematocrit of the PKD-L and PKD-H groups were significantly lower ($P < 0.05$) than the control group (Table 20). Signs of anemia were not apparent, no infected fish had a hematocrit below 24, a level that Wedemeyer & Yasutake (1977) suggest as the lower limit for healthy trout. The mean Hct of the PKD-M fish was also lower than that of control fish, however, it was not significant

Table 18. Prevalence of parasite infection and severity of lesions in rainbow trout (AR-RTH) with proliferative kidney disease.

Sample Date	Interstitial PKX Stage (No. infected / No. examined)	(%)	M or H LESION* (No. infected / No. examined)	(%)	Intraluminal Stage (No. infected / No. examined)	(%)
June 18	18/21	(86)	2/21	(10)	0/21	(0)
July 6	12/20	(60)	5/20	(25)	2/20	(10)
July 15	9/20	(45)	8/20	(40)	1/20	(5)
July 29	5/20	(25)	2/20	(10)	2/20	(10)
August 11	0/10	(0)	0/10	(0)	3/10	(30)

* Infected fish were classified into three groups (L = light, M = moderate, or H = heavy) by the severity of microscopic lesions of the kidney.

Table 19. Effect of proliferative kidney disease on fork length (FL), weight (W), and condition factor** (K-FACT) of rainbow trout (AR-RTH).

GROUP+	FL (cm)	W (g)	K-FACT
CONTROL			
Mean	16.5	54.2	1.1804
SD	1.5	15.3	0.1269
n = 60			
PKD-L			
Mean	16.0	47.2	1.1266*
SD	1.6	14.6	0.0848
n = 24			
PKD-M			
Mean	15.5	42.6	1.1180
SD	1.7	12.3	0.0744
n = 9			
PKD-H			
Mean	16.5	52.8	1.1880
SD	0.7	5.1	0.1102
n = 8			

\+ Fish were either exposed (PKD) to the infectious agent or maintained on well water (CONTROL). Infected fish were classified into three groups (L = light, M = moderate, or H = heavy) by the severity of microscopic lesions of the kidney.

* Significantly differently from controls at $P < 0.05$.

** Condition factor= $100*W/L^3$.

Table 20. Effect of proliferative kidney disease on hematocrit (Hct),leukocrit (Lct), hemoglobin concentration (Hb), and mean corpuscular hemoglobin concentration (MCHC) in rainbow trout (AR-RTH).

Group*	Hct	Lct	Hb (g/dl)	MCHC
CONTROL				
Mean	36.7	1.0328	6.8	18.5
SD	3.9	0.3605	1.5	3.5
n = 40				
PKD-L				
Mean	34.3**	1.2948+	5.2+	16.2**
SD	4.3	0.3198	0.9	3.5
n = 21				
PKD-M				
Mean	34.1	1.1300	5.6**	15.6**
SD	6.4	0.3677	2.1	5.3
n = 8				
PKD-H				
Mean	33.3**	1.8497+	5.6**	16.6
SD	4.7	0.6175	1.6	3.3
n = 7				

* Fish were either exposed (PKD) to the infectious agent or maintained on well water (CONTROL). Infected fish were classified into three groups (L = light, M = moderate, or H = heavy) by the severity of microscopic lesions of the kidney.

** Significantly different from controls at $P < 0.05$.

\+ Significantly different from controls at $P < 0.01$.

($0.05 < P < 0.10$). Hemoglobin content and MCHC were lower in infected fish than controls. The MCHC for the PKD-H group while lower than that of the controls, was not significance ($0.05 < P < 0.10$). Ferric iron was not observed in spleen sections from either control or infected fish. Infected fish demonstrated a higher leukocrit (PKD-L and PKD-H significant at $P < 0.01$) than the controls (Table 20). An increased number of circulating monocytes and granulocytes were observed in the PKD-H fish. The relative percentage of the other leukocytes did not vary between groups (Table 21).

Serology. The severity of histopathological lesions appeared to correlate with changes in several serological parameters. The PKD-H fish had higher calcium and magnesium plasma concentrations than the control fish (Table 22). The magnesium concentrations of the PKD-M fish ($P < 0.01$) were also higher. While plasma chloride concentrations in the PKD-H and PKD-L group were greater than the controls, these differences were not significant ($0.05 < P < 0.10$).

The mean plasma cortisol values (SD) of the infected fish did not differ ($P < 0.05$) from the controls (μg/100 ml): CON ($n = 14$)= 1.42 (0.72), PKD-L ($n = 12$) = 1.61 (2.36), PKD-M&H ($n = 12$) = 1.69 (1.73). Plasma concentrations of glucose did not vary significantly ($P < 0.05$) among the groups.

Table 21. Effect of proliferative kidney disease on the relative percent of lymphocytes, thrombocytes, monocytes and granulocytes counted from 100 non-erythrocyte cells per bloodsmear of rainbow trout (AR-RTH).

GROUP+	THROMBOCYTE	GRANULOCYTE	LYMPHOCYTE	MONOCYTE
CONTROL				
Mean	8.4	2.4	89.0	0.2
SD	8.0	1.7	7.4	0.1
n = 20				
PKD-L				
Mean	12.6	3.4	82.6	1.4*
SD	4.7	2.4	6.8	2.6
n = 12				
PKD-M				
Mean	6.4	2.4	90.5	0.7
SD	4.5	1.9	5.8	0.8
n = 8				
PKD-H				
Mean	6.5	5.5*	85.7	2.3*
SD	6.2	2.7	4.2	2.6
n = 8				

\+ Fish were either exposed (PKD) to the infectious agent or maintained on well water (CONTROL). Infected fish were classified into three groups (L = light, M = moderate,or H = heavy) by the severity of microscopic lesions of the kidney.

* Significantly different from controls at $P < 0.05$.

Table 22. Effect of proliferative kidney disease on plasma chloride, calcium, glucose, magnesium and total protein concentrations in rainbow trout (AR-RTH).

Group*	Chloride mmol/l	Calcium mg/dl	Glucose mg/dl	Total Protein g/dl	Magnesium mg/dl
CONTROL					
Mean	128.20	10.67	83.73	3.41	2.09
SD	3.76	0.54	9.86	0.35	0.15
n = 15					
PKD-L					
Mean	130.23	10.45	78.15	3.49	2.23
SD	3.14	1.75	11.73	0.25	0.28
n = 13					
PKD-M					
Mean	126.75	10.89	89.67	3.53	2.30+
SD	2.68	0.73	15.03	0.42	0.11
n = 12					
PKD-H					
Mean	131.00	11.16+	79.78	3.89+	2.38+
SD	2.31	0.27	9.98	0.32	0.22
n = 9					

* Fish were either exposed (PKD) to the infectious agent or maintained on well water (CONTROL). Infected fish were classified into three groups (L = light, M = moderate, or H = heavy) by the severity of microscopic lesions of the kidney.

\+ Significantly different from controls at $P < 0.05$.

Plasma samples were separated into six fractions (F1-F6) by electrophoresis (Table 23). The PKD-H fish had a significant reduction in pre-albumin (F1) and albumin (F2) fractions and an increase in the globulin fractions (F4) and (F6). The strong cathode migration of F6 indicates that it contained immunoglobulin (Busch, 1978). While the total protein concentration of the PKD-H fish was higher than that of the controls (Table 22), the albumin/globulin (A/G) ratio was significantly lower.

Nitroblue tetrazolium assay. The twelve control fish used in the NBT trials had a mean absorbance of 0.280 (SD = 0.109). The NBT assays were conducted while the infected fish were in the later stage of PKD. Only five of fifteen PKD fish tested were of moderate or heavy rating. Their mean absorbance of 0.219 (SD = 0.054) was not significantly ($P < 0.05$) different from the control group. Because of the interstitial hyperplasia of the PKD fish, a 3 to 4X dilution of the pronephros cell suspension was needed in order to match the cell density ($2.0 - 2.8 \times 10^5$ cells/$mm^{3)}$ of the control group.

Table 23. Effect of proliferative kidney disease on the protein distribution* of rainbow trout (AR-RTH)** plasma separated by electrophoresis.

Fraction	Mean Relative Percent (SD)			
	Control (n = 15)	PKD-L (n = 10)	PKD-M (n = 8)	PKD-H (n = 8)
F1	8.7 (3.6)	8.0 (4.2)	6.0 (4.5)	4.0 (2.8)+
F2	42.5 (6.0)	41.5 (6.9)	44.5 (4.4)	33.6 (8.3)+
F3	10.8 (2.7)	9.0 (1.5)	9.0 (1.6)	10.9 (3.1)
F4	20.5 (5.0)	19.8 (1.7)	19.0 (6.3)	26.7 (5.3)+
F5	10.0 (2.3)	9.5 (1.9)	11.0 (7.2)	11.7 (3.3)
F6	9.2 (4.1)	12.0 (5.1)	10.8 (4.9)	17.9 (7.1)+
A/G++	1.1 (0.3)	1.0 (0.1)	1.1 (0.2)	0.6 (0.2)+

* Cellulose acetate membrane electrophoresis at 250 V for 25 min. Total protein value and densitometer scanning pattern used to calculate the relative percentage for each fraction.

** Fish were exposed (PKD) to the infectious agent or maintained on well water (CONTROL). Infected fish were classified into three groups (L = light, M = moderate, or H = heavy) by the severity of microscopic lesions of the kidney.

\+ Significantly different from controls at $P < 0.01$.

++ Albumin/globulin ratio.

Plaque forming cell assay. In both the first (June 29) and second (July 20) PFC trials, the PFC responses of fish with PKD were higher than that of the controls (Table 24). Microscopic examination of PKX infected trout on June 29 and July 20 showed they were in the early and peak stages of the disease, respectively. Trout in the PKD group of the final (August 15) trial did not differ from the controls in their PFC response. However, only 2 of 12 assayed had histological lesions and no parasites were detected. Attempts to detect agglutinating antibody to SRBC in the serum of PFC fish were unsuccessful.

Vaccination and bacterial challenge. The fish from the PKD group were more resistant to V. anguillarum than the control group in all three challenges (Tables 25 & 26). Examinations of kidney sections from the non-vaccinated PKD fish in the first (June 16) challenge and the sham vaccinated PKD fish of the second (July 7) and third (August 9) challenges showed that they were in the early, peak, and recovery phases of PKD respectively. The prevalence of infection (PKX) and the number of fish with moderate - to - heavy rating (M/H) for the PKD challenge survivors were as follows: (June 16) PKX = 66.7 % (8/12) and M/H = 16.7 % (2/12); (July 7) PKX = 50.0 % (8/16) and M/H = 31.3 % (5/16); (August 9) PKX = 6.9 % (5/72) and M/H = 1.4 % (1/72). Intraluminal sporogonic forms were the only stages of PKD observed (15/72) in rainbow trout surviving the last (August 9) challenge.

In both the second and third challenges, the PKD-bacterin fish showed similar survival rates as their control cohorts (Table 26). None of the non-challenged fish (PKX or control, sham or bacterin) from either the second (July 7) or third (August 9) challenge died during the study.

Table 24. Effect of proliferative kidney disease on the plaque forming cell (PFC)* response of rainbow trout (AR-RTH).

Group**	Date	PFC/10^6 Leukocytes (SD)	
CONTROL (n = 16)	June 29	18.4 (2.7)	
PKD (n = 8)	June 29	36.0 (9.5)	P < 0.01
CONTROL (n = 12)	July 20	9.7 (1.7)	
PKD (n = 5)	July 20	16.1 (4.0)	P < 0.01
CONTROL (n = 10)	August 15	59.4 (18.1)	
PKD (n = 2)	August 15	39.3 (11.7)	NS

* Fish were injected intraperitoneally with 0.1 ml of 10% sheep red blood cells and isolated leukocytes assayed in agar for the PFC response 14 d later.

** Fish were either exposed (PKD) to the infectious agent or maintained in well water (CONTROL).

NS Not significantly different from controls at P < 0.05.

Table 25. Effect of proliferative kidney disease on survival of rainbow trout (AR-RTH)* following challenge** by Vibrio anguillarum.

	No. mortalities / No. exposed (%)		
	PKD	CONTROL	
	13/50 (26)	21/50 (42)	$P < 0.05$

* Trout were either exposed (PKD) to the infectious agent or maintained on well water (CONTROL). The average weight of the trout in the PKD and CONTROL groups were 43.3 g (SD = 12.2) and 39.8 g (SD = 9.7) respectively.

** Trout were challenged by a 15 min bath of a 12 h V. anguillarum culture at 3×10^4 cells/ml. Mean water temperature during the 12 d challenge was 16.4 C.

Table 26. Effect of proliferative kidney disease on the response to bacterin vaccination* and challenge with Vibrio anguillarum in rainbow trout (AR-RTH)+.

Challenged July 7			Challenged August 9		
PKD	CONTROL		PKD	CONTROL	
Bacterin Groups					
5/56 (9)**	2/56 (4)	NS	6/56 (11)	1/56 (2)	NS
Sham Groups					
13/56 (23)	39/56 (70)	P < 0.01	31/56 (55)	46/56 (82)	P < 0.01

* Trout were vaccinated by a 15 min bacterin bath containing 10^6 cells/ml formalin-killed, V. anguillarum. The fish were challenged after 20 d at 16 C with a 15 min bath of a 18 h V. anguillarum culture. Mortalities were monitored for 12 d. The challenge dosage and mean water temperature for July 7 and August 9 were 1.2 x 10^6 cells/ml and 16.0 C ;and 6 x 10^4 cells/ml and 15.1 C respectively. Sham-vaccinated groups received identical treatment except a 0.4 % formalized BHI broth was used instead of bacterin. No mortality occurred in the non-challenged fish from either treatment groups

\+ Trout were either exposed (PKD) to the infectious agent or maintained on well water (CONTROL) . The average weights of the PKD and CONTROL groups of trout on July 7 and August 9 were 49.2 g (SD = 14.0), 52.0 g (SD = 8.4) and 88.9 g (SD = 22.4), 92.1 g (SD = 18.9) respectively.

** no. mortalities / no. exposed (%)

NS Not significantly different from controls at P < 0.05.

DISCUSSION

Infections with the PKX parasite stimulated the defensive mechanisms of affected trout. Fish which recover from PKD are resistant to reinfection. Changes in homeostasis and the development of clinical signs in PKX infected trout are correlated with the severity of renal lesions.

Survivor resistance. Sentinel fish demonstrated that the seasonal occurrence of PKD at ARH is dependent on the presence of the infectious stage from April to October or November. In Europe, PKD infections occur from May through November when water temperatures are 15 C or above (Ferguson & Ball, 1979; Clifton-Hadley et al., 1984). Seasonal patterns of infectivity have been reported for several myxosporeans. This seasonality may result from endogenous cycles of the parasites, availability of susceptible hosts or environmental influences on the host-parasite relationship (Ahmed, 1973; Uspenskaya, 1978; Wyatt, 1978; Wolf & Markiw, 1984). In our study, PKD infection was not directly linked to the temperature of the water in which the fish were held. Temperature may influence the emergence of the infectious stage in the environment.

There were no differences in time of infection between fish held in ambient (7 - 13 C) and 17 C water. Temperature is a strong environmental influence on the host-parasite relationship as it modulates both the defensive response of the teleost host and the activity of the parasite (Finn & Nielson,

1971; Avtalion et al., 1976; Sypek & Burreson, 1983). Ferguson (1981) suggested that PKD is temperature-dependent and that low temperatures inhibit the development of the disease. Clifton-Hadley et al. (1986) reported that PKX-infected trout held at 9 and 12 C had little to no renal swelling while those held at 15 and 18 C had swollen kidneys characteristic of the disease. Water temperature may influence both the emergence or presence of an infective stage in the environment and the progress of the disease once the fish is infected. The combination of these two factors will determine the severity of the disease and subsequent development of resistance to reinfection.

Disease resistance seems to depend on recovery from clinical disease rather than just exposure to PKX. This was shown by examinations of the ARH production trout in Group B. Although exposed to the infectious stage upon their introduction to ARH in October 1984 , parasites were not detected until June 1985. The onset of first infection of Group B trout, coincided with that of the Group C fish which were presumptively naive to PKX as they had been introduced to ARH at a time when the infectious stage was absent (December). Thus it appears that no resistance to infection was acquired by Group B fish during their brief exposure to the parasite in October. The lack of infection in the Fall may have been due to both declining water temperature and possibly reduced concentration of the infectious agent. In contrast, resistance to disease was apparent in trout which had recovered from PKD.

The resistance to reinfection with the interstitial PKX stage in survivors from the 1984 outbreak (Group A) in 1985, correlates with the observations of Ferguson & Ball (1979). Although sporogonic stages were seen in the lumina of kidney tubules of the Group A fish in 1985, these were most likely residual parasites from the 1984 outbreak. The high prevalence of infection and severity of lesions in the control yearling fish exposed to parasite during the same period, corresponds to observations by D'Silva et al. (1984). They concluded that age alone is not a protective factor against PKD. This supports the presumption that the resistance to PKD found in the Group A yearlings was due to an acquired immunity to the parasite. Similar results were obtained when the degree of disease resistance was examined by artificial challenge of PKD survivors.

Survivor trout (AR) injected IP with a kidney suspension containing parasites were very resistant to interstitial PKX infection, similar to the Group A yearlings exposed to a water source with the infectious stage. The few sporogonic stages seen in the lumina of kidney tubules in both AR-PKX and AR-MEM groups were most likely residual parasites from the 1985 outbreak. The survivor fish used in the 1986 (autumn) IP injection experiment had come from a population at ARH which had shown no clinical or histological signs of interstitial PKX infection during the 1986 summer epizootic. It is assumed that resistance to PKX had developed from infections during from the

1985 outbreak and possibly enhanced by a second exposure to the infectious stage during the summer of 1986. While the prevalence of infection in the naive control trout injected with PKX was similar to that of naive yearlings infected by natural exposure, the intensity of infection and renal lesions were less severe.

The IP challenge tended to produce focal lesions in the naive DS-PKX fish in contrast to the diffuse granulomatous lesions commonly observed in natural infections. This difference in lesions may be due to several factors. First, trout used in the study were large (avg wt 143 g) in relationship to the single dose (0.2 ml) of PKX injected per fish. Therefore, the number of parasites which gained access to the kidney was probably lower than that which occurs in a natural epizootic where continuous exposure to the infective stage is found. The vegetative reproduction capacity of the parasite is not fully understood and may be limited in a healthy host. Second, the period of parasite invasion and subsequent host response for the injected fish was short in comparison to fish undergoing continuous field exposure. The limited renal lesions in the DS-PKX fish was exemplified by the similarity in hematological and serological parameters between this group and the control DS-MEM.

Mean hematocrit of the DS-PKX fish (35.9) was significantly lower than the DS-MEM controls (42.1), however, this value was not below the normal range of 24 - 43 % for rainbow trout (Wedemeyer & Yasutake, 1977). No obvious

difference in serum calcium, total protein, albumin or chloride concentrations was observed between infected and control fish of the same strain. The serological data obtained from the study can only be used to examine broad trends between the treatment groups due to the pooled nature of the serum samples. Serum had originally been collected with the intention of using it in agglutination assays. These assays were not done because of difficulties in producing a purified antigen. Direct correlation between the individual severity of renal lesions and pathophysiological changes was not possible as the sampled kidneys were placed in a single fixative container for each treatment group. From the limited amount of physiological data collected it appears that infection does not necessarily result in a disease state.

Resistance to reinfection appears to be quite solid in fish which have recovered from clinical PKD. The results of the IP challenge of survivor trout indicate that this state of resistance is not due solely to non-specific defence mechanisms. Intraperitoneal injection of the parasite bypasses the external defenses of the epidermis, the digestive enzymes of the gut, and mucus components such as lysozyme, complement and protease. If an immune response is responsible for the resistance observed in both the field exposure and experimental infection studies, it is possible that immunoglobulin from survivor trout could reduce parasite infectivity.

Effect of immune serum on parasite infectivity. Incubation in serum from presumptively immune PKD survivors did not reduce the infectivity of the PKX parasite. It was hypothesized that specific immunoglobulin to PKX in this "immune" serum would induce either lytic action by the complement system or increased host phagocyte efficiency due to opsonization. External antigen(s) of certain protozoan ciliates can induce a protective immune response from teleosts (Hines & Spira, 1974; Goven et al., 1980). Jones & Woo (1987) report that incubation (3 h at 10 C) with immune trout serum did not reduce the infectivity of the blood flagellate Cryptobia salmositica. In comparison, the PKX parasites were incubated for 30 min at 25 C. This incubation period and temperature was chosen as they had been documented in trout to be effective in both lytic activity and opsonization (Nonaka et al., 1981; Griffin, 1983; Sakai, 1984b).

Whether the "immune" serum contained specific immunoglobulin to parasite antigen(s) is unclear. It is possible that PKX may have the ability to either mask or sequentially alter its antigenic profile as observed with certain trypanosomes (Cohen, 1982). Attempts at using "immune" sera for both indirect fluorescent antibody staining of PKX imprints and double gel diffusion assays with infected kidney homogenates as antigens were unsuccessful (unreported data). However, Olesen & Jorgensen (1986) report that trout infected with PKD had IgM concentrations which averaged 34 % of the total serum protein in

comparison to 5 - 6 % of the healthy control trout. This hypergammaglobulinemia suggests that the parasite does evoke a humoral response. Successful host defense may be more depended on cellular immune mechanisms rather than humoral components.

Blood parameters and immune status. Both the experimental (DS) and natural (AR) infection studies focused on the isolated physiological effects of PKD on rainbow trout. The effects on fish physiology of such variables as fish size, age, genetic background, consistency of sampling protocol and the laboratory environment were reduced to a minimum in the studies. In both studies, neither the infected fish nor their respective controls appeared to be undergoing a stress response as both plasma cortisol and glucose levels were within the normal range for salmonids (Schreck & Lorz, 1978; Wedemeyer, 1972), and these analytes did not differ between the groups. Kent & Hedrick (1987) observed similar cortisol levels in PKX infected fish. It appears that PKX parasites were the primary influence on fish health in the study groups.

No bacterial or viral agents were isolated from fish of either study. It seems that PKX parasites were the sole pathogens in the case of the AR-RTH fish. While Chloromyxum sp. and trematodes were observed in the kidneys of DS-RTS fish, these parasites did not appear to exert an effect on the pathology or pathophysiology caused by PKD. The effect of PKX infection on growth was closely monitored in the laboratory

studies.

No discernable trend was observed between infection and condition factor (K-FACT). Fish in the PKD-H group from both studies had similar condition factors as their respective controls. While the K-FACT of the DS-RTS (PKD-L) and (PKD-M) fish were slightly higher than the MEM controls, the opposite occurred in the field exposure study. The biological significance of these K-FACT differences is questionable, as none were below the normal (K) value of 1.11 for rainbow trout (Piper et al., 1982). It appears that subclinical PKD does not significantly alter growth.

In the experimental infection study, PKX parasites were first detected in the kidney at 3 - 4 wk post-injection (P.I.). The peak of infection occurred at 6 - 7 wk P.I. and was followed by a recovery phase. While the time of first infection could only be estimated for the AR trout, the disease course paralleled that of the infected DS trout. Kent & Hedrick (1986) reported a similar disease pattern for experimentally infected trout held at 15 C. Few fish in our study demonstrated the classic PKD sign of swollen, mottled kidneys as only 18% (8/44) of the AR-PKX and 8% (7/90) of the DS-PKX fish were rated as PKD-H. As in the experimental survivor resistance study, a single IP injection of a homogenate made from PKX-infected kidneys into yearling trout tended to produce focal lesions. The minor effect of PKX infection on blood parameters revealed the adaptive capacity of trout to adverse conditions.

The PKX infected DS-RTS fish had reductions in erythrocyte number (RBC) and hematocrit (Hct), however, hemoglobin concentration (Hb) did not change. Changes in erythrocytes did not correlate closely with the severity of renal lesions in kidney sections. While the AR-RTS fish infected with the parasite had a decrease in Hct and Hb, a relationship to pathological rating was not apparent. Other workers have reported more dramatic hematological changes in PKD affected fish. Clifton-Hadley et al. (1987a) observed that packed cell volume (=Hct) declined in direct relationship to the degree of renal swelling. Fish in their "no renal changes" group and the control AR-RTH fish in our study had very similar erythrocyte values. The drop in Hct and Hb reported by these workers was much more severe than that seen in the AR-RTH infected fish groups. Fusiform crystals (up to 50 μm in length), reported to be composed of hemoglobin, have been observed in intravascular PKD lesions (Smith et al., 1984; Clifton-Hadley et al., 1987a), however, no such crystals were seen in the present study. It is possible that a severe infection may induce the formation of hemoglobin crystals. Hoffmann & Lommel (1984) reported a mean hematocrit of 28 % and a mean hemoglobin concentration of 2.4 mmol/L in trout with severe clinical signs of PKD. Infection with the PKX parasite appeared to affect certain erythrocyte parameters in both AR and DS fish.

Mean cell hemoglobin concentration (MCHC = Hb / Hct x 100) is calculated from two parameters which can be measured with

some accuracy. This index did not change in the infected DS-RTS yearling fish, however, the infected AR-RTS fish did show a decrease in their MCHC. A reduction in erythrocyte hemoglobin concentration may be responsible for the drop in this index, however, hypochromasia was not observed in blood smears. No obvious change in erythrocyte size or morphology was observed in the DS-RTS (PKD-L) blood smears to support the increased MCV. Control fish from both studies had hematological and serological values consistent with normal trout. Blood parameters of the control DS-RTS (MEM) fish, with the exception of the higher values for WBC count and MCHC, and lower total protein values, were within the 96 percentile range of values for the Shasta strain given by McCarthy et al. (1975). The blood parameters of the AR-RTH control fish were also consistent with values reported in the literature. The mean values for hemoglobin, hematocrit, chloride, glucose, and total protein were within the normal ranges stated by Wedemeyer & Yasutake (1977) for rainbow trout. Both the leukocrit and MCHC were also within normal ranges (McLeay & Gordon, 1977; McCarthy et al., 1975). Plasma concentrations of magnesium and calcium were higher than those values generally reported in the literature (Hille, 1982). These differences could have been related to age of fish, diet, and water chemistry.

Subclinical PKD resulted in a reduction in the number of circulating erythrocytes but did not produce signs of anemia even in fish rated as PKD-H. No increase in the number of

immature erythrocytes or a change in their staining characteristics was observed in the blood smears of either the DS-RTS or AR-RTH infected fish. Therefore, it did not appear that erythropoiesis or hemoglobin biosynthesis were severely affected by subclinical disease. Pale gills is a clinical sign of anemia commonly associated with PKD (Clifton-Hadley et al., 1984). There are three general categories for anemia; hemorrhagic (arising from blood loss), hemolytic (excess erythrocyte destruction), and hypoplastic (decreased erythrocyte production). Hoffmann & Lommel (1984) categorized the anemia seen in PKD to be hemolytic in nature. Clifton-Hadley et al. (1987a) considered it to be primarily hypoplastic with some hemolytic elements. The replacement of kidney hematopoietic tissue due to PKD lesions suggests that the observed anemia is primarily hypoplastic in nature. Hemolytic anemia tends to produce hemosiderin deposits in vascular organs such as the spleen. Hoffmann & Lommel (1984) reported seeing hemosiderin deposits in the spleens of infected trout. Clifton-Hadley et al. (1987a) also observed such deposits but in only a few infected fish which prompted the authors to speculate that severe hemolysis was not involved in producing anemia. Aguis (1979) reported that PKD did not result in any increased iron deposition in the kidney, liver, and spleen. In the present study, no significant ferric iron deposits were observed in Prussian Blue stained spleen sections. However, few fish showed signs of severe anemia. Erythrocyte retention in the spleen

without hemosiderin deposition has been reported in anemic fish infected with Renibacterium salmoninarum , the agent responsible for bacterial kidney disease (Bruno & Munro, 1986). Bacterial kidney disease produces granulomatous lesions in the kidney which are similar to those observed in PKD. It is possible that a high level of hemolysis must occur before serum haptoglobin capacity for hemoglobin-binding is overwhelmed and hemosiderin deposition becomes significant. Hemoglobin can also be eliminated through the kidney, however, the loss of nephrons seen in clinical PKD would minimize this route of excretion. Increased erythrocyte removal, depressed erythropoiesis or blockage of immature erythrocytes may have been involved in the reduction of erythrocytes observed in the AR-RTH infected fish. Clifton-Hadley et al. (1987b) speculated that kidney hyperplasia may have prevented immature erythrocytes from entering the circulation after their formation in the hemopoietic regions. Proliferative kidney disease affected leukocyte values to a greater degree than those of the erythrocytes.

There appeared to be a direct correlation between the severity of renal lesions and an increase in leukocrit. Both the DS-RTS and AR-RTH fish in the PKD-H rating groups had higher leukocrits than the controls, PKD-L, or PKD-M groups. Kent & Hedrick (1987) reported a mean leukocrit value of 2.5 ($\pm$ 0.6) in PKX infected trout which is greater than the leukocrits observed in the present study. An increase of circulating granulocytes and monocytes in the AR-RTH fish occurred as the severity of renal

lesions increased. No such change was seen in the infected DS-RTS fish. Both Clifton-Hadley et al. (1987a) and Hoffmann & Lommel (1984) observed similar increases in circulating phagocytic cells. Weinreb (1958) reported that trout injected with turpentine had an initial rise in circulating neutrophils and speculated that this was a normal inflammatory response. Ellassaesen & Clem (1986) report that handling stress induced neutrophilia in catfish. Serological parameters were also affected by subclinical PKD.

Minor changes in plasma constituents were seen in both the infected AR-RTH and DS-RTS fish. The slight increase in plasma chloride concentrations seen in the AR-RTH (PKD-H) fish and the decreased values of all infected DS-RTS fish were probably of little biological significance as they remained within the normal range for rainbow trout (Wedemeyer & Chatterton, 1970). Impaired divalent ion excretion, as evident from increased plasma concentrations of magnesium and calcium, were observed in the AR-RTH (PKD-H) fish and to a lesser extent in the PKD-M fish. The infected DS-RTS trout showed a similar increase in plasma magnesium levels, however, calcium concentrations did not differ from those of the MEM controls. Albumin retention efficiency, as evidenced from low plasma albumin levels, was also impaired in fish from both studies with a PKD-M or PKD-H rating. Tubule loss and injury may have been the cause of both the increase in divalent ions and the drop in albumin. Kent & Hedrick (1987) report that tubule density drops in PKD affected

fish due to interstitial hyperplasia. Although the albumin level declined in the AR-RTH (PKD-H) and DS-RTS (PKD-M and PKD-H) fish, elevated globulin fractions resulted in an increased total protein concentration and low A/G ratio. This indicates that a responsive increase in globular protein production was occurring while renal filtration was impaired due to PKD.

Workers have separated four to ten fractions using cellulose acetate membrane electrophoresis (Evelyn, 1971; Ingram & Alexander, 1977; Busch, 1978). Five fractions (DS-RTS) and six fractions (AR-RTH) were separated from the plasma samples. As a pre-albumin fraction was rarely observed in the DS-RTS fish plasma proteins graphs, "F1a" was established to be a combination of the albumin and pre-albumin (when present) fractions. The albumin fraction(s) of the DS-RTS fish did not drop as dramatically as the infected AR-RTH trout. This may have been due to the focal lesions seen in the kidneys of the infected DS-RTS fish which would have allowed for regions of functional nephrons with normal ion excretion and protein reabsorption capabilities. Plasma protein fractions F4 and F6 were elevated in the AR-RTH (PKD-H) trout as had been observed by Scott (1984) in clinically ill fish. The strong cathode orientation of F6 indicates that immunoglobulin was present, while F4 may represent acute phase proteins such as C-reactive protein and alpha-2 macroglobulin (Winkelhake & Chang, 1982; Ellis, 1981). The DS-RTS infected trout showed a similar plasma protein profile as the AR-RTS fish except fraction F4 (=AR-RTH

F5) also increased. Olesen & Jorgensen (1986) noted that PKD affected trout had IgM levels which were 34 % of the total serum protein compared to 5 - 6 % in their control fish. The strong inflammatory response to the parasite led us to examine the effects of infection on both specific and non-specific defensive mechanisms.

Sub-clinical infections with the PKX parasite appeared to stimulate certain non-specific defenses and did not interfere with its immune response. Phagocytes such as macrophages and neutrophils are important components in the fish's non-specific defenses (Ellis, 1981). Olivier et al. (1985) demonstrated that stimulation of salmonid macrophages play a major role in protection from bacterial pathogens. Salmonid phagocytes are reported to share many characteristics of higher vertebrates such as complement receptors, a myeloperoxidase killing system, and increased activity to opsonized targets (Johnson & Smith, 1984; Kanner & Kinsella, 1983; Griffin, 1983; Sakai, 1984b). The increased oxidative metabolism of activated phagocytes can be identified by the reduction of nitroblue tetrazolium dye (NBT) to blue formazan and by chemiluminescence (CL) assays (Baehner & Nathan, 1968; Scott et al., 1981). No significant differences between the NBT or CL responses of infected versus control trout were observed. This indicates that the phagocytic cell capability of the fish is not impaired by subclinical infections. There was a high degree of individual fish variation in CL responses irrespective of the treatment group.

Angelidis et al. (1987) reported that trout with "gross lesions of PKD" had a lower CL response than cohorts without lesions. Granulomatous lesions could impair the physiology of the kidney leukocytes used in their study due to localized hypoxia and nutrient deficiencies. The infected DS-RTS trout did not have macroscopic lesions of any great proportions (only slight swelling and mottling) and therefore may not have experienced the same degree of physiological imbalance as the fish used by Angelidis et al. (1987). The NBT assay was chosen for the field exposure study due to the high variability encountered in the CL trials. Infected trout in both the CL and NBT experiments had increased numbers of leukocytes in their kidneys when compared to the control fish without interstitial hyperplasia. Anterior kidney cells of salmonids are reported to show an increase in phagocytosis when the fish are subjected to stress (Peter & Schwarzer, 1985). An increase in both the number and state of activation might explain the results of challenges of PKD and control trout with V. anguillarum.

In both studies, PKX infection of non-vaccinated and sham-vaccinated trout tended to increase their resistance to V. anguillarum challenge over that of the control fish. This was especially true of fish in the early stages of PKD. This increase in non-specific defense was apparent even in fish with moderate to heavy disease ratings. As the state of PKD regressed, the difference in challenge survival between PKX-infected and control fish decreased. In the 60 d (P.I.) DS-RTS

challenge, there was no significant difference in survival between the control and PKD sham-vaccinated fish. The prevalence of infection and severity of renal lesions indicated that the challenge survivors (PKD groups) were in the early recovery stage of PKD. The sham-vaccinated PKD fish (AR-RTH) of the third (August 9) challenge had significantly ($P < 0.01$) lower cumulative mortality than their control cohorts, however, the difference in the mortality between PKD versus control fish (82 % - 55 % = 27 %) had dropped in comparison to the second (July 7) challenge (70 % - 23 % = 44 %). Histological examination of the PKD-infected, V. anguillarum challenge survivors of the second (July 7) challenge showed that these fish were just beginning to recover from PKD while the PKD-infected fish of the third (August 9) challenge were in the late recovery phase. Therefore, it appears that the presence of the PKX parasite in the kidney interstitium stimulates the non-specific defenses of the fish and that this heightened degree of resistance may return to a basal level as the fish recovers from PKD. Neither phagocyte or lymphocyte function appeared to be impaired by subclinical PKD.

No difference in the protection afforded by immunization with a V. anguillarum bacterin was seen between PKD and control groups. The bacterin groups had a much higher challenge survival rate than their sham-vaccinated cohorts. This indicates that the bacterin conferred protection from challenge under the conditions of the studies and that PKD infection did

not impair the fish's immune response. The Vibrio bacterin and the bath vaccination method were chosen due to their successful use in both research and commercial endeavors (Gould et al., 1979; Kaattari & Irwin, 1985). Tatner & Horne (1986) demonstrated that trout will absorb ^{14}C - labeled V. anguillarum bacterin within 10 s of contact. Kaattari & Irwin (1985) reported that coho salmon leukocytes were stimulated by V. anguillarum O-antigen extract to produce plaque forming cells. Veile et al. (1980) demonstrated that adoptive transfer of both plasma and leukocytes from immersion-vaccinated donor trout to naive recipients would confer protection from challenge. Angelidis et al. (1987) stated that vaccinated (0.2 ml I.P. Vibriffa™ injectable) trout which had gross PKD lesions were more susceptible to V. anguillarum challenge than cohorts without gross lesions. This statement was based on the observation that a higher number of fish which survived the challenge were without gross lesions. It is unclear from the report what other factors such as environmental or previous health conditions might have been involved in the challenge mortality seen in these commercially-reared trout. Normal lymphocyte function and antigen processing in fish from our study with subclinical PKD was also indicated by the PFC trials.

The plaque forming cell (PFC) assay was used to investigate the primary immune response of PKX-infected trout. While there were some discrepancies in the results, it appears that infection with the PKX parasite does not cause a reduction in

this immune response. There was no significant difference between the PKX and MEM injected groups (DS-RTS) at both 36 and 60 d post PKX injection. In contrast, the AR-RTH PKD fish in the early PFC trials (June 29 and July 20) had significantly higher PFC responses than the controls. Kidney sections from these fish showed both interstitial parasites and renal lesions. As only two fish in the third (August 15) trial had PKD-like microscopic lesions (no parasites were seen in any of 12 PKD fish assayed), no conclusions can be made from this trial. One possible factor in the different PFC responses observed between the DS-RTS and AR-RTH fish, could be that the larger DS-RTS fish were not stimulated by PKX infection to the same degree as the smaller AR-RTH fish. As mentioned previously, the DS-RTS fish tended to have focal lesions in the kidney from the I.P. PKX injection and showed a lesser degree of change in blood and serologically parameters than the naturally infected AR-RTH fish. Another size related possibility may be that the SRBC dosage given to the DS-RTS fish was not large enough to produce an optimum PFC response.

Sheep red blood cells were selected as the antigen for the PFC trials on the basis of results by other workers (Blazer, et al., 1984; Jayaraman et al., 1979). Although this antigen proved to be adequate for the studies, stronger PFC responses have been reported using other preparations such as O-antigen extracts (Kaattari & Irwin, 1985). The selection of a 14 d post-injection assay time was based on the PFC kinetics studies of

Anderson et al. (1979b). They concluded that the maximum PFC response in trout held at 11 C occurred at 16 d post exposure to Yersinia ruckeri O-antigen. As the water temperature in our experiments was higher (15-16 C), it was felt that a shorter time to sample could be employed and still have confidence that the sample period occurred at peak PFC response. One drawback of the PFC method is the high individual variation which occurs within the test groups. Unlike laboratory mice which are nearly identical due to genetic and environmental control, hatchery-reared rainbow trout are much more heterogenous. Anderson et al. (1986) used an in vitro immunization/spleen culture technique to avoid this problem. One fish would provide its own control cells in this protocol. Unfortunately, only in vitro effects can be examined by this method. Another drawback of the PFC technique is that the time consuming and laborious aspects of this assay reduces the numbers of fish which one investigator can handle in a trial. Two PFC assay methods were used in the course of this research. While the Cunningham technique was more sensitive than the agar slide method, there were problems in sample volume regulation and stability of plaques with the former technique.

Angelidis et al. (1987) reported that although protection from challenge was lower in immunized trout with gross PKD lesions, there was no significant difference between trout with and without lesions in their anti-Vibrio antibody titer. This result indicates that B-cell populations could still respond to

the antigen even though the fish were clinically ill. The bacterin and PFC results of the present study also indicate that lymphocyte function and antigen processing is not impaired by PKX infection.

This study has demonstrated that fish which recover from infection by the PKX parasite are immune to reinfection, however, in vitro incubation of the parasite in survivor serum is not protective in artificial challenges. Subclinical infection results in a stimulation of the fish's non-specific defenses and does not impair its ability to mount an immune response. Abnormal serological and hematological changes occur only in those fish with substantial renal lesions. Previous field studies which emphasized pathophysiological changes associated with severe clinical signs (Ferguson & Needham, 1978; Hoffmann & Lommel, 1984) do not portray the physiological state of fish with moderate to mild infections. It appears that the isolated effects of subclinical PKD do not significantly suppress the defense mechanisms of the fish. Therefore, infection by the PKX parasite is not a sole predisposing factor to secondary infections and affected fish can recover from the disease without serious problems if other environmental and biological stressors are reduced.

SUMMARY & CONCLUSIONS

1) The ability to infect trout held in a water supply which historically produces PKD epizootics is related to the seasonal presence of the infectious stage and not the effect of water temperature on the development of the disease in the host.

2) A strong resistance to PKD reinfection by either artificial or natural challenge is present in fish which have recovered from previous infection by the PKX parasite.

3) Exposure to water containing the infectious stages of PKX without subsequent infection does not induce resistance to reinfection.

4) Intraluminal forms of the parasite may be present in the lumina of kidney tubules at least 13 mo following the initial infection.

5) There appears to be a positive relationship between the dosage of PKX injected (quantity of kidney suspension containing the parasite/ weight of recipient fish) and the intensity of histological lesions and infection in the kidney. This indicates that parasite multiplication

within the fish may be limited by endogenous factors or by host-response.

6) Co-incubation with serum from resistant donors did not reduce the infectivity of PKX prior to injection of kidney suspensions into recipient trout. This suggests that serum factors alone are not responsible for resistance.

7) Severe lesions of the kidney must be present before significant changes occur in blood and serological parameters.

8) Infection with the PKX parasite under laboratory conditions stimulated non-specific defense responses and the production of globular plasma proteins (including immunoglobulin).

9) PKX infection did not affect the specific immune response of the infected trout.

10) Infection with the PKX parasite under laboratory conditions did not invoke a significant acute stress response as evident by changes in either plasma cortisol or glucose concentrations.

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Appendix 1

Chemiluminescence Reagents

Adapted from Scott & Klesius (1981)

I. Luminol (5-amino-2,3 dihydro-1,4 phthalazinedione)

Sigma Chemical Co., P.O.Box 14508, St. Louis, MO 63178

Potassium hydroxide	0.780 g
Boric acid	0.618 g
Luminol	0.014 g
Distilled Water	10 ml

8 mM stock solution was then diluted 1:1000 with HBSS without Phenol Red 4-12 h prior to an assay. The working solution was stored in an aluminum foil-wrapped tube (prevent light penetration) at 4 C.

II. Zymosan (Sigma Chemical Co.)

Twenty milligrams of zymosan was boiled for 30 min in 2 ml PBS (pH 7.0). The suspension was centrifuged at 600 x G for 5 min and the supernatant removed by pipette. Five milliliters of pooled trout serum was added to the zymosan pellet, vortex mixed, and incubated for 30 min at 25 C. This mixture was then centrifuge as above and the pellet washed once with PBS. The washed pellet was suspended in 4 ml of HBSS without Phenol Red to obtain a suspension of 5 mg/ml zymosan.

Appendix 2

Serum calcium (Ca), total protein (TP), albumin (Alb), and chloride (Cl) values from five fish pool samples in experimentally challenged* control trout (DS-RTS/RTKJ).

GROUP	DAYS POST INJECTION	Ca (mg/dl)	TP (g/dl)	Alb (g/dl)	Cl (meq/l)
PRETRIAL	0	10.0	4.0	1.54	118
PKX	18	10.5	4.8	1.71	125
MEM	18	11.4	4.7	1.46	126
PKX	28	9.7	4.3	1.65	132
MEM	28	10.5	4.6	1.54	107
PKX	42	10.8	4.4	1.54	111
MEM	42	10.3	5.5	1.90	104
PKX	57	10.8	4.7	1.77	113
MEM	57	8.9	4.5	1.62	122

* Fish were injected intraperitoneally with 0.2 ml of either minimum essential media (MEM) or a kidney suspension containing the parasite (PKD).

Appendix 3.

Serum calcium (Ca), total protein (TP), albumin (Alb), and chloride (Cl) values from five fish pool samples in experimentally challenged* survivor trout (AR-ELT).

GROUP	DAYS POST INJECTION	Ca (mg/dl)	TP (g/dl)	Alb (g/dl)	Cl (meq/l)
PRETRIAL	0	10.0	4.2	1.90	108
PKX	18	10.5	4.1	1.69	126
MEM	18	11.1	4.6	1.90	135
PKX	28	11.6	4.7	1.77	113
MEM	28	10.3	3.8	1.81	122
PKX	42	10.3	4.3	1.71	120
MEM	42	10.0	4.6	1.85	125
PKX	57	10.0	4.3	1.62	111
MEM	57	10.3	5.4	1.77	122

* Fish were injected intraperitoneally with 0.2 ml of either minimum essential media (MEM) or kidney suspension containing the parasite (PKD).