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A MANUAL OF - FISH PATHOLOGY

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PREFACE

One of the main limitations of aquaculture production is the susceptibility of farmed fish to diseases due to farming practices or external factors such as pollution, climate change or even changes in the dynamics of product transactions in this industry. However, it is important to better understand and characterize those involved in the disease outbreak process as these result in huge economic losses in the aquaculture industry. High-throughput technologies such as proteomics can be an important characterization tool, especially in the identification of pathogens and virulence mechanisms related to host-pathogen interactions in disease research and diagnosis, contributing to the control, prevention, and treatment of diseases in farmed fish. The important role of proteomics is also maximized by its holistic approach to understanding pathogenesis processes and fish responses to external factors such as stress or temperature, making it one of the most promising tools for fish pathology research.

CHAPTER 1

INTRODUCTION

The demand for animal protein for human consumption is increasing due to an exponential increase in world population. Aquaculture is becoming an increasingly important source of protein for human consumption as it is an industry capable of providing solutions to feed a rapidly growing population and alleviate poverty in many countries. To achieve this, the scale of aquaculture production and the range of species farmed have increased significantly over the past two decades. Live production always carries the risk of loss from infectious diseases, as farming practices in aquaculture make farmed fish more susceptible to diseases from a variety of bacterial infections, viruses, parasites and fungi than wild fish. Also, the trend towards increasingly dense production systems, disturbances in the balance of ecosystems associated with pollution and climate change, and the expected increase in international transactions in aquaculture products and their derivatives have all contributed to changing the dynamics of interaction between organisms and infectious agents, and people. This affects the replication and multiplication rates of pathogens, leading to a wider geographic spread of pathogens and an increase in species affected by outbreaks. This makes disease outbreaks a significant barrier to this industry, with a significant impact on the quality, safety and volume of fish produced worldwide, which can result in foreclosure from market access and economic or significant costs to the producer.

1.1 INTRODUCTION

The ocean is a vast ecosystem and has a great variety of fish. Fish has high economic value and fills the food shortage. Marine fisheries are important to the economy and well-being of coastal communities as they provide food security, employment opportunities and livelihoods (Bell, 1978; Delgado et al., 2003). Global marine fisheries produced 81.5 million tonnes of fish in 2014 and directly employed 34 million people in fisheries in 2014 (FAO, 2016). In India, marine fisheries produced 34,18,821 tons of fish in 2014 (FAO, 2016).

Ecological and environmental parameters such as biotic and abiotic factors play an important role in fisheries biomass. Abiotic factors such as seasonal variations, ocean currents, water temperature, oxygen levels, nutrients, pollution, sewage, industrial effluent and toxic materials, and biotic factors such as predators, diseases, pathogens and parasites affect fish diversity and

biomass. The marine environment contains a variety of physicochemical and biological parameters that can stress fish and cause outbreaks of disease when they are at levels greater than acceptable (Roberts, 1989). Due to increased air and water temperature, which can increase vector organism reproduction (Freed et al., 2005) and pathogen population growth (Woodhams et al., 2008), and also accelerate transmission rates through the proliferation of infectious disease stages (Freed et al., 2005). Fish diseases are one of the most important problems for fisheries biologists today.

The marine environment with a variety of physico-chemical and biological parameters, if the concentration of these parameters exceeds the marked acceptable limits, can stress fish and lead to outbreaks. The results improve vector organism proliferation and increase the growth rate of the pathogen population by increasing the water temperature, and also accelerate transmission rates by proliferating the infection stages. Natural populations of fish show tumors in almost all tissue systems. Tumor is a common disease in which cells are aggressive, invasive, and sometimes metastatic. Tumors are caused by both viruses and non-infectious carcinogens (chemicals).

For several authors, aquaculture disease outbreaks are the result of a complex web of interactions in aquatic systems between the organism produced, multiple environmental and zootechnical aspects, and potential pathogens that pose a unique set of health challenges for aquatic organisms, as illustrated in Figure 1 .

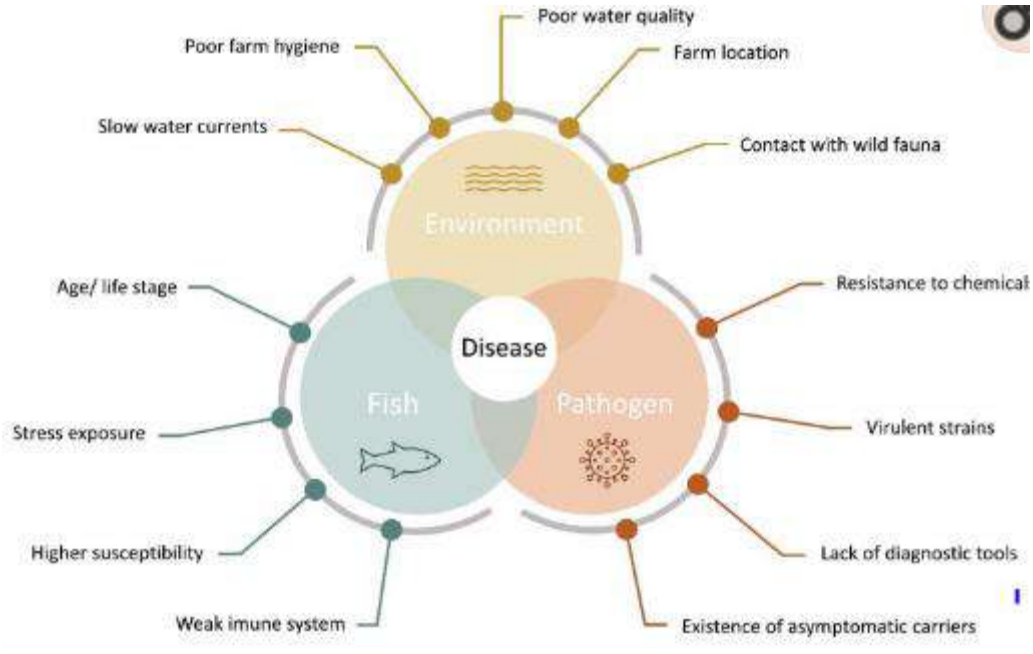


Figure 1.1 Diagram of diseases in aquaculture showing the main pathogen scoring factors and host-pathogen interactions involved in fish disease outbreaks.

To address infectious pathologies in farmed fish, approaches such as epidemiological studies on important aquatic animal health areas such as transboundary and emerging aquatic animal diseases, animal health surveillance and the development of biosecurity programs should be implemented. These are critical for monitoring disease prevalence, early detection of exotic and emerging diseases, and improving quality management at aquaculture farms.

However, in order to obtain appropriate epidemiological models, animal health surveillance and biosecurity programs need to integrate environmental information and information from different domains such as pathogenesis, disease diagnosis, disease resistance, physiological response to pathogens, characterization of pathogens, characterization of host immune system responses, disease biomarkers and response of the body on products for the treatment of diseases.

The amount of data from different sources and the increasing frequency and severity of reported marine diseases require the implementation of new diagnostic tools for faster and more efficient diagnosis. Therefore, several scientific advances in aquatic health continue to bridge the gap with veterinary medicine, and new techniques in optics, analytical chemistry, molecular biology and omics are becoming a reality, bringing a multitude of benefits to the aquaculture industry. Proteomics techniques are one of these new tools and one of the most interesting approaches to fish health management, epidemiology and disease research. Proteomics refers to the methodology concerned with the study of the total complement of proteins expressed in a particular state of an organism or cell population. The proteome, or complete protein complement of the genome, is a highly structured unit in which proteins perform their cellular functions with temporal and geographic specificity in physical or functional association with other proteins or biomolecules. Mass spectrometry (MS)-based high-throughput proteomics methods enable the measurement of multiple properties for thousands of proteins, including their abundance, tissue distribution, subcellular localization, post-translational modifications, and protein-protein interactions. Proteomics-based approaches can therefore provide unique insights into the cellular regulation of fish in response to pathogens and during disease progression, and furthermore enable rapid and sensitive pathogen detection and identification.

This manuscript will provide detailed information on the use of proteomics in various aspects of disease, with particular emphasis on the role of stress and well-being in disease and the importance of pathogen identification and host-pathogen interactions for diagnosis and disease characterization.

1.2 HEALTH, STRESS AND WELFARE OF FISH

Although fish are the most consumed animal, they rarely evoke the same concern for their welfare as other vertebrates. Scientific research on the welfare of fish is still at an early stage compared to other land animals intended for human consumption. In part, this lack of consideration is due to the discrepancy between public perceptions of their intelligence and scientific evidence, and the lack of a consistent definition of the term. Nevertheless, most definitions mainly consider a mood-based approach and a function-based approach. The first takes into account the animal's emotional state, while welfare is defined as the absence of negative feelings and the presence of positive feelings. The second definition focuses more on the animal's biological, physiological and health perspective, while good welfare is defined as the fish's ability to cope and adapt to its environment while maintaining homeostasis. Although fish health status provides objective criteria for assessing animal welfare, it does not provide a complete picture. Good health is essential for good welfare, but does not necessarily mean that the fish is in good condition. On the other hand, poor health, ie the animal's reduced ability to function normally, cope with stressful conditions and prevent disease, generally implies/results in a poor state of well-being. For example, dead fish as a result of disease are a source of infection and affect water quality. In addition, chemical treatments for specific outbreaks can also induce some level of disturbance in fish. It is important to note that a healthy animal in an optimal environment can also be suddenly attacked by an acute infection that affects its well-being. For example, in fish that are raised in cages, pathogens are naturally introduced into the environment. In most cases, it is often the poor social status itself that leads to poor health due to poor housing conditions. In summary, then, health and well-being are closely related, and poor well-being can be interpreted as both a cause and a consequence of poor health. This section focuses on health as the cornerstone of assessing fish welfare and the impact of stressors on disease resistance, and provides an overview of the latest approaches to studying the relationship between specific diseases/conditions and welfare.

In aquaculture, inadequate housing conditions, even standard housing practices, are daily stressors in farming systems. The allostatic stress imposed on animals can impair the

functioning of immune mechanisms, thereby promoting disease and threatening fish welfare (Figure 2). For example, drastic changes in water temperature (from 27 °C to 19–23 °C or 31–35 °C) decreased immune response and pathogen resistance in Mozambique tilapia (*Oreochromis mossambicus*). More recently, using a transcriptomic approach, culture density of Nile tilapia (*Oreochromis niloticus*) has been shown to have a significant impact on susceptibility to the oomycete *Saprolegnia parasitica*. However, the link between livestock-related stress and disease is not so straightforward. For example, acute stressors have been reported to enhance or decrease certain innate immune responses in fish. In contrast, chronic stressors have mainly been indicated as immunosuppressants. From a productivity perspective, fish health is often interpreted as "disease-free" since any disease state is not ethically or economically acceptable to the industry. Therefore, disease prevention and eradication are crucial aspects of a fish farm to ensure the sustainability of production. Creating optimal well-being conditions, regularly monitoring health parameters and reducing stress are necessary steps to achieve this goal.

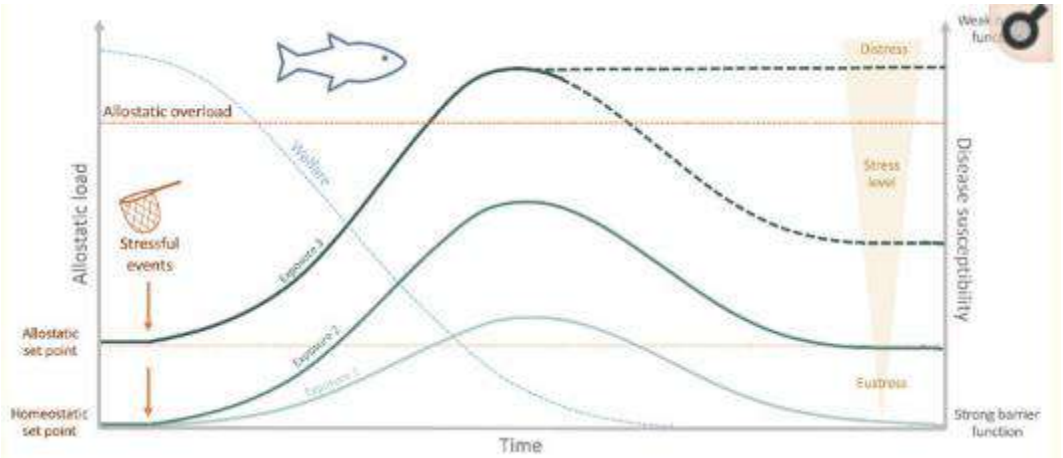


Figure 1.2 Interaction between well-being, allostatic stress, disease susceptibility and repeated/chronic stress experiences assessed by fish. Stress stimuli can induce adaptive (eustress) or maladaptive (distress) allostasis. If the stressor persists, recovery to the original homeostatic state (homeostatic set point) may be incomplete. In this case, a newly defined setpoint is set for future adaptation (allostatic setpoint). As a result, wellness status decreases with time and the stress experienced. Cumulative adaptive load (allostatic load) therefore consists of the beneficial stress events to which the fish may be subjected, while allostatic overload represents the condition where the stress exceeds the organism's natural ability to regulate, which can produce a state of no-recovery. At this stage, primary barrier function is

severely compromised, increasing susceptibility to disease, which can lead to disease and eventual death.

Stress is viewed as a state of threatened homeostasis that is being restored through a complex web of changes in physiological systems (allostasis). Like all other vertebrates, fish elicit a general response to perceived stress, called the physiological stress response, which allows the individual to adapt and cope with both predictable and unpredictable changes in their environment (eustress). As the primary response, cortisol and catecholamines are released into the bloodstream, triggering a series of downstream responses. In fact, stress is not necessarily harmful or synonymous with impaired well-being. On the contrary, in the short term, it is an essential adaptation to ensure the best chance of survival. However, when allostatic overload is reached, usually as a result of a prolonged, repeated, and/or unavoidable stressor, maladaptive effects such as impaired growth and/or impaired reproductive and immune functions occur (Figure 2). In this case, these are largely associated with reduced well-being and can affect the health and survival of the fish (distress). The questions raised here are the costs of this acclimatization and why stress increases disease susceptibility in fish. First, in terms of energy costs, the adaptive physiological response required to counteract disrupted homeostasis requires a significant amount of energy. This means that if some of the fish's energy is devoted to meeting the challenge, there are fewer resources available for other energy-consuming biological functions, such as feeding. B. Certain mechanisms of the defense repertoire: the epithelial barriers and the immune system. Regarding immune responses, multiple mechanisms are immediately activated to directly respond to the challenge. These include elevations in inflammatory markers, hormone release, and expression of acute-phase proteins. Even if a fish has successfully adapted to the stressor for a period of time, these energy stores will eventually be depleted if the stressor persists. Consequently, the total consumption of energy stores leads to allostatic overload and the fish may become unable to adapt, leading to immunosuppression, disease, and even death in more severe disorders (Figure 2). In addition, several studies have also demonstrated the influence of stressful rearing conditions on the function of the epithelial barriers, i.e. the mucous and epidermal surfaces of the skin, gills and intestines, which are the main lines of defense against pathogens and pollutants. Damage to these barriers inevitably leads to impaired resistance against diseases. Changes in these barriers have been reported in Atlantic salmon (*Salmo salar*), cod (*Gadus morhua*), and rainbow trout (*Oncorhynchus mykiss*) exposed to various acute stresses. In addition, impairment of gut barrier function has also been observed in Atlantic salmon reared under low

dissolved oxygen levels. These disorders have been primarily linked to elevated cortisol levels, although various other hormones such as catecholamines, endogenous opioids, pituitary hormones and serotonin also play a role. In fact, cortisol is known to play an immunomodulatory role, inhibiting certain components of the immune system and enhancing others, such as: B. the induction of apoptosis, the change of differentiation patterns, the inhibition of the release of cytokines and the inhibition of the migration of immunocytes. Nevertheless, the cortisol response can vary between different species and even between individuals (coping styles) and can be influenced by several other parameters (e.g. degree of domestication, age, nutritional status, severity of stress, u immune status. A detailed description of how the endocrine immune response is constructed and the mechanisms driving these changes in immune regulation are beyond the scope of this review, for which the authors refer to recent publications.

Deepening our scientific knowledge of the mechanisms related to stress, health and fish welfare is vital to the sustainable aquaculture industry. In recent years, more advanced high-throughput technologies, as in the case of proteomics, have been successfully applied in aquaculture research, including for the study of fish diseases and welfare, providing a holistic understanding of the molecular events underlying the physiological response to stress and valuable insights into different proteins involved in inflammatory processes and immune responses. Proteomics studies in fish are primarily targeting the liver, however blood plasma and mucus are becoming increasingly important, especially from an immunological point of view since skin mucus is one of the most important defensive barriers in fish and plasma acts as a mirror/detector for physiological or pathological conditions. Important applications of proteomics in this field relate to the study of the effects of certain diseases and parasites on protein abundance and modification, and the study of host-pathogen interactions. For example, collaborative studies evaluating changes in the proteome of fish exposed to a specific pathogen after exposure to a rearing stressor are rare. However, existing proteomics studies showing that aquaculture and environmental stressors clearly modulate immune function in fish indicate that these technologies are already promising sensitive approaches to study this relationship.

It is easier to see the importance of 'stress' factors in fish diseases than in diseases of other farmed or wild species. The word *stress* has different meanings for different groups of workers. As originally defined by Selye (1950), it was "the sum total of all physiological responses by which an animal attempts to maintain or restore normal metabolism in the face of a physical or

chemical force". Brett (1958) gave a definition that correlated more easily with fish health. He proposed that "stress is a step caused by an environmental or other factor that extends an animal's adaptive responses beyond the normal range or that disrupts normal function to such an extent that chances of survival are greatly reduced ". However, the measurement of this stress, while essential to many aspects of pathogenesis research, has not been performed reliably, perhaps because the concept is applied to an assemblage of very diverse phenomena. Changes that occur in response to environmental stress are known as the *general adaptation syndrome* (GAS). These are essentially non-specific physiological and biochemical changes that take place in three phases: 1. The *alarm reaction*. 2. The *resistance stage,* where adaptation to achieve homeostasis under the changed circumstances takes place. 3. The *exhaustion stage,* when adaptation is no longer adequate and homeostasis is not achieved. The changes that occur during SGA are neither species nor stress specific: anoxia, infection, fear, forced exercise, anesthesia, and many other stressors cause similar responses in higher and lower vertebrates. The ensemble of responses to each individual stressor is different, made up of some responses specific to that particular stressor and others (e.g., stress) that are the usual changes in the SGA, regardless of the type of stress. Therefore, as pointed out by Pickering (1981, 1998), GAS in its pure form is not easy to define because of the difficulty in distinguishing the general from the specific. Events involving GAD are mediated by hormonal and neural responses. The production of adrenocorticotropin hormone (ACTH) and corticosteroids leads to the retention of Na+ and Cl- ions, while K+ ions are excreted; there is an increase in blood sugar and nitrogen metabolism; the thyroid gland is stimulated and the production of thyroxine increases; and in the blood, lymphocytopenia and neutrophilia are generated. The sympathetic nervous system also responds, resulting in spleen contraction, increased respiratory rate, and increased blood pressure. Sumpter (1997) has elegantly reviewed the role of the hypothalamic-pituitary-internal (HPI) axis in GAS. He points out that not all fish respond to stress by exhibiting a fixed pattern of endocrine changes. For example, in fish previously exposed to pollutants, the response is altered (Hontela *et al.* 1992) and is both sensitive and reactive to stressors such as husbandry practices, disease and xenobiotics. Slicher (1958) and McLeary (1975) studied the effects of "stress hormones" in fish. They showed that when injected into fish in small doses, cortisol and ACTH cause leukocytopenia, while high doses of ACTH cause leukocytosis. The effect of small doses of ACTH on circulating leukocyte counts could be mimicked by cold shock, which consists of immersing the fish in a pool of cold water for a short time. The stimulation of the HPI axis by stress mainly results in increased plasma cortisol levels, and the physiological consequences of this in fish include the series of events collectively known as the *stress*

response (Sumpter 1997). Injection of cortisone into fish has been shown to cause delayed leukocyte infiltration of inflammatory lesions and inhibition of wound healing response. ACTH and corticosteroids injected into teleosts cause depopulation of the lymphoid tissues in the kidney and spleen, although little change in thymic tissue has been reported (an interesting contrast to the response in mammals, where cortical thymocytes are destroyed by ACTH). Even in control fish injected with saline, there was an appreciable amount of necrosis in the lymphatic tissue of the kidney and spleen that was not apparent in uninjected fish. In addition to stimulating corticosteroid production, hypothalamic stimulation of chromaffin tissue, the location of which varies between species but is located in the anterior stroma of the kidney in salmonids, results in an adrenergic response and the release of catecholamines. These produce secondary effects, mainly on circulation, osmoregulation and energy.

THE CELLULAR RESPONSE TO STRESS

A until recently understudied feature of SGA in higher animals or fish and shellfish is the "cellular stress response" (Locke 1997). Cells typically respond to stress via changes in gene expression, leading to upregulation of blood and tissue levels of a group of proteins collectively known as heat shock proteins (HSPs). HSP molecules produced in response to stressful conditions are not only key components of the early response to stressors, but are also integral to host defenses against neoplasms and chronic pathogens, and may become a major avenue for the development of new vaccines (Srivastava 2002).. HSPs, also called extrinsic chaperones, are a series of highly conserved proteins of varying molecular weights (from *approximately* 16 kilodaltons (kDa) to 100 kDa) that are produced in all cellular organisms when subjected to cellular stress (Welch 1993). Although originally recognized in the fruit fly (Ritossa 1962) where it was found that specific genes are upregulated when their cells are exposed to heat, it is now recognized that the response is universal for all cells and those in fish and other vertebrate stressors such as anoxia, ischemia, toxins, protein degradation, hypoxia, acidosis and microbial damage also lead to their upregulation (Chiang *et al.* 1989; Welch 1993). The intracellular homologues of HSPs, also called chaperones, constitutive chaperones or heat shock relatives (HSCs), are also found in the cytoplasm of normal, non-stressed cells and account for 5-10% of the total protein in these healthy growing cells (Pockley 2003)... Although it was originally discovered that HSPs are upregulated when fruit fly cells are exposed to heat (Ritossa 1962; Tissieres *et al.* 1974), it is now recognized that HSCs are normally mainly produced and located in the cytosol, nucleus and mitochondria. They are

universal to all cells and are essential for a variety of homeostatic functions, including maintaining protein structure and folding, supporting and repairing damaged elements of the cytoskeleton, supporting intracellular protein production and folding, enzymes and hormone receptors, and maintaining Lipoprotein membranes mitochondria and cell walls (Beckman *et al.*, 1990). The role they play in maintaining cell membrane lipoproteins, which must be modified to ensure their proper functioning, is particularly important with regard to fish and other thermal cycling when temperature changes occur. However, when cells are stressed, whether from cold, heat, ultraviolet (UV) radiation, toxins, pathogens, nutritional deficiencies, protein degradation, hypoxia, acidosis, microbial damage, or other cellular stress, the newly formed HSC constituents HSPs upregulate, which can be detected in cells at concentrations two to three times higher than the constituent chaperones and in fluid tissue (Locke 1997; Chiang *et al.* 1989). Hence the term *stress proteins* (or SPs), a broader term, is also used to describe them (Locke 1997). Since the induction of HSPs is not limited to thermal stressors but can be induced by any stressor if the intensity is sufficient (Feder & Hoffman 1999), the upregulation of HSPs is generally described as part of the cellular stress response. The regulation of gene transcription of heat shock proteins is mediated by the interaction of heat shock factors (HSFs) with heat shock elements in promoter regions of the gene (Voellmy 1994; Pockle, 2003). Animal and plant HSFs show striking structural similarity, but there are significant differences in the complementation and activity of HSF family members in different groups of organisms (Feder and Hofmann 1999). For example, many insect groups such as fruit flies have only one HSF (Clos *et al.*, 1990), while fish and other vertebrates have three or four (Scharf *et al.* 1998; Pirkkala *et al.* 2001). In vertebrates, HSPs are classified into several families and named according to their function, sequence homology, and molecular mass in kDa. The families mainly include Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and several smaller Hsp groups. Many members of the HSP families have homologues called HSCs that are expressed in the cell under normal stress-free conditions. These play a fundamental role in regulating normal protein synthesis within the cell. HSP families such as HSP 90 and HSP 70 are essential for the folding and assembly of other cellular proteins (Gething & Sambrook 1992), and these and other molecular chaperones are also involved in regulating partitioning kinetics between folding, translocation and aggregation, and play a broader role Role in relation to immune, apoptotic and inflammatory processes (Ellis 1990; Moseley, 2000; Srivastava 2002; Pockley 2003). When cells are exposed to chemical or biological toxins, whether iatrogenic in origin or resulting from microbial activity, denaturation of proteins within the cell or on the cell surface occurs due to weakening of polar bonds and consequent exposure of

hydrophobic groups. This leads to errors in protein folding and aggregation (Wedler 1987). Hightower (1991) suggested that this is a particularly important means by which cellular stressor effects occur and coined this mechanism of damage with the epithet *proteotoxicity*. HSP 90 and HSP 70 synthesis, which increases dramatically under such cellular stresses, serves to protect or facilitate early repair of these damaged proteins. If stress impairs the non-specific and specific immune response of an animal, an increased susceptibility to infection is to be expected. Such an increase has been observed in bony fish and has been extensively studied by Wedemeyer (1997). Whatever the mechanisms, once the degradation stage is reached in the SGA, gut and environmental microorganisms that are harmless to healthy fish can invade the host. Therefore, great caution must be exercised in the interpretation of bacteriological findings in these fish, since opportunistic perimortem invaders can often be isolated in significant numbers from internal organs. However, the presence of microorganisms with real pathogenic potential in an environment is much more likely to lead to full-scale disease outbreaks when the fish are under stress (ie during changes in adaptive response). At such times, the added burden of invading pathogens is a cumulative and increasing counter-effect to the fish's attempts to restore homeostasis. Ambient temperature is probably the most important stressor affecting the balance between the host fish and the environment. Each species has a normal temperature range and an absolute minimum and maximum beyond which it cannot survive. When temperature approaches these extremes, pathogen invasion is likely to occur, especially at peak temperatures. Even within the normal acceptable temperature range, temperature can be a significant cause of disease, but it's usually a sudden change, rather than the temperature *itself*, that is the stressor. Temperature changes can affect the rate of reproduction of microorganisms, as can the amount of dissolved oxygen in the water, the rate of excretion of metabolites or, more importantly, the speed at which host defense mechanisms and the production of antibodies can respond. The general principle emerging from studies of the effects of temperature on phagocytosis, inflammation, wound healing, and toxic and septic microbial diseases suggests that the rate of host defense response is very similar for all species studied at a given temperature. However, the higher the temperature within the acceptable range for a species, the faster the various reactions (Roberts 1975a). An interesting manifestation of this general principle was shown by Anderson and Roberts (1975) in wound healing in tropical and temperate bony fish. They showed that similar wounds healed at similar rates in both species where the true tempera ranges overlapped. The advantages of the maximum healing rate possessed by the white mountain minnow, its tropical species, at the peak of its range over the Atlantic salmon, its temperate species, simply reflected its ability to

withstand a range of higher temperatures. Many bony fish species, but particularly salmonids, experience high mortality rates during the spawning season. These are usually associated with fungal and bacterial invasion. In the Pacific salmon, these deaths are complete by the first (and only) spawn. The stressors at sexual maturity are primarily hormonal, but hunger and, in migratory species, travel fatigue and osmotic effects also play a role. The most important hormonal change is the sharp increase in the concentration of 17-hydroxycorticosteroids in the blood, which is histologically reflected in increased activity of the pituitary and adrenal glands, although upon reaching full maturity the pituitary gland shows degenerative changes, while the adrenal gland is degenerative and hyperplastic (Robertson & Wexler 1962a,b). Hyperglycemia associated with hyperplasia of Brock's human bodies (islets of Langerhans) was reported in many other species at this time, as well as in salmonids (Love 1970). Gonadotropin production increases with sexual maturity and it has been shown that, at least in rainbow trout, testosterone is converted to biologically active 5-dihydro-testosterone in the epidermis (Hay *et al.* 1976). This could explain the large changes that occur in the structure and susceptibility to infection of this tissue in the sexually mature male (Pottinger *et al.* 1995). Other changes that occur that increase the susceptibility of spawning salmonids include changes in the intimal coronary arteries and glomerular degenerative changes.

1.3 PATHOLOGY

Pathology is the scientific study of disease, is a branch of medical science. Pathology encompasses the structural and functional changes of disease, primarily with regard to the study of organs, tissues, and body fluids. The ultimate goal of pathology is diagnosis of disease and etiology, a fundamental goal that leads to successful therapy and disease prevention (Underwood, 2004). It is usually divided into two main branches, clinical pathology and pathology or a combination of both, called general pathology. Clinical Pathology: Laboratory analysis of blood, urine, and tissue samples to study and diagnose diseases. Anatomopathology: study and diagnosis of the disease based on the study of a biopsy or a surgically removed autopsy.

1.4 FISH PATHOLOGY

Fish pathology is the study of diseases of fish and crustaceans and is a branch of veterinary medicine. Diseases in fish are closely related to environmental stress. In the wild, they usually have some degree of freedom to change their environment. The anatomy and physiology of

fish are modified mainly according to the two main ecological factors that control their existence: the aquatic environment and the Poi Kilo Therm's inability to control its temperature. These factors are also of paramount importance in determining the chain of events following pathological changes such as microbial infection, traumatic injury, or nutritional deprivation.

Marine and estuary fish diseases have received considerable attention in recent years. This is partly because marine fish disease surveillance has been recommended as a useful tool for monitoring biological impacts (McIntyre and Pearce, 1980). Disease and parasitic infestations are a threat to fish diversity and it is important to identify pathogens and parasites that pose a risk to biodiversity (Smith et al., 2006). External diseases of marine fish have been recognized as having potential for monitoring biological impacts, including fin rot, ulcers and epidermal neoplasms, with growing interest in their occurrence and frequency.

1.5 WHOLESALE PATHOLOGY

The different age (size) group of Indian oil sardine was observed. Most adults were seen, followed by breeders and elders. Normal fish show no lesions on the body surface (Fig. 2). Gross observation clearly shows that the sardine tumors varied in location, appearance, consistency, and size. Multicolored tumor masses covering the entire exterior (head, maxilla and mandible, mouth, eye, tongue, gills, both sides of the operculum plates on outer and inner regions, both sides of the flanks, all fins and caudal peduncle) and interior (stomach) be noted, kidney, liver, ovary, testis, and celomous cavity) organs of Fishes (Figs. 2–16). The neoplasms ranged in diameter from 1.5 to 21.5 mm. Most tumors were reddish to pale pink in color and opaque in nature, while some were black, white, opalescent, and translucent. Most tumor masses were unilobed and some were multilobed with varying consistency ranging from soft, jelly-like flesh to hard bone. Fleshy tumors were seen almost on the outer regions of the body, but bony tumors arose from skeletal organs such as the jaw, operculum, and fin rays. There was no gross evidence of metastasis, but some of the neoplasms were locally involved. Partially degraded tumors were also observed (Figure 17). Radiographic analysis of fish with sardine tumors clearly showed multiple unilocular and radiopaque bone lesions and an enlarged tissue mass of the stomach and retroperitoneum merging with part of the digestive tract (Figs. 18 and 19). The normal structural arrangements of other visceral organs were disturbed.

Figure 2. Sardinella longiceps of different ages; old (A), adult (B) and laid (C). Scale in centimeters.

1.6 SYSTEMATIC PATHOLOGY

Apart from Ferguson's excellently presented text, there are few integrated reviews of the range of pathological processes that can occur in the various organ systems of bony fish. The information given in this section is therefore largely based on clinicopathological observations and not on detailed experimental studies. In particular, there is a lack of information about systems such as the nervous and endocrine systems, a gap that urgently needs to be addressed. Much of the information is presented in a different form elsewhere in this text. However, it is provided here on a systemic basis, since the pathologist approaching a new case thinks primarily in terms of the physiological systems involved. He can then logically assess how he might be affected by the many different types of pathogens that might be responsible for the observed lesions and make an informed diagnosis.

1.7 TUMO

The tumor is a cellular proliferative disease in which the cells are infiltrating, aggressive, invasive, and sometimes metastatic. The terms "neoplasia" and "new growths", especially when referring to the lower animal, are difficult to define precisely. Meissner and Warren (1971) defined a neoplasm as "a growth disorder characterized primarily by incessant, abnormal, and excessive cell proliferation." Prehn (1971) defines neoplasia as "that form of hyperplasia which is caused at least in part by an inherently hereditary abnormality in the cells involved".

Neoplasia is a disease in which genetically engineered cells evade normal growth regulation. Important concepts in the definition of neoplasia include: (i) the presence of an abnormal mass with growth not coordinated with normal tissue; and (ii) the persistence of overgrowth after cessation of the lesion-eliciting stimulus (Willis, 1967). Abnormal growth is to some extent structurally and functionally host independent because neoplastic cells are partially devoid of the controls that act to regulate and limit normal cell growth (Sirica et al., 1989). The persistence of growth after removal of the neoplasm evoking factor indicates that the neoplastic trait is a change in DNA structure or expression inherited by successive cell generations.

Several morphological features distinguish neoplasms from normal tissue and other types of lesions. Neoplastic growth is not controlled by the same mechanisms as normal tissue control. This results in persistent, expanding, or infiltrating growth without the architecture of normal tissues.

Neoplasms usually form grossly visible masses, but this is not an integral part of the concept of neoplasia. Neoplasms exhibit varying degrees of abnormality in cellular appearance and growth rates, and functional differences are usually evident between neoplastic tissue and related normal tissues. Tumor is a common disease in all eukaryotic multicellular organisms. Tumors were only reported in humans and later in all major types of tumors found in all domestic and wild animals, including fish. Neoplasms have been reported in many species of aquatic organisms including copepods (Crisafi and Crescenti, 1975; Vanderploeg, 1998; Bridgeman et al., 2000; Jagadeesan and Jothibabu, 2016), sea lions (Moore and Stackhouse, 1978; Sato et al., 2002), seals (Brown et al., 1975), dolphins (Sanchez et al., 2002), aquatic lizard (Schmidt, 1977), turtles (Herbst et al., 1999; Work et al., al., 2004).), seahorse (Boyla et al., 2014), crab (Brock and Lightner, 1990), shrimp (Overstreet and Devender, 1978), lobster (Shields and Small, 2013), and fish (Bell, 1793; Schlumberger and Lucke, 1948 ; Wellings, 1969; Groff, 2004).

1.8 FISH TUMO

Fish oncology is important not only because of the impact of neoplasms on fish and fish populations, but also because fish are good models to advance our understanding of neoplasms. Neoplasms grow on the skin and other visceral organs of fish. Neoplasms occur in many species of fish, both farmed and wild. Epidermal neoplasms are found in freshwater and marine environments worldwide (Harshbarger and Clark 1990; Dethlefsen et al. 2000). The first occurrence of tumors in fish was reported as early as 1563 when farmed carp were infected with papillomatosis in Europe (Hofer, 1906). The first known report of a tumor in wild fish appeared much later, in 1793. Thus, the earliest scientific documentation of a diseased fish was of a species of Chaetodon with tumor-like growths in the bones (Bell, 1793). Over 50 types of tumors have been reported in over 1000 fish species worldwide.

The presence and high prevalence of tumors in farmed and captive fish populations has been reported in almost all organ tissue systems and has been widely reported by scientists and naturalists (Wellings, 1969; Mawdesley Thomas, 1971; Harshbarger, 1977; Moore et al., 1996). Because of the characteristic appearance of tumors and their apparent pathologic nature, more than 50% of fish tumors are skin related (Anders and Yoshimizu, 1994; Mawdesley-Thomas, 1971). The prevalence of papillomas in certain fish populations can be very high. In one study, up to 55% of Pleuronectidae studied from the Pacific coast of North America had one or more papillomas. These occurred in younger fish, mainly on the pigmented side (Stich et al., 1977). These are considered by some researchers to be degenerate parasites, but there is evidence that in the case of Pacific flatfish papillomas they are most likely transmitted by a virus (Peters et al., 1983).

In fish, skin tumors that are externally visible are among the most commonly reported. Epidermal neoplasms are more common than tumors of visceral organs in freshwater and marine environments worldwide (Harsh Barger and Clark, 1990; Dethlefsen et al., 2000). Although epidermal neoplasms are common in many species of fish and the incidence of tumors is similar between species, there are many differences in the etiology of the disease between the species studied. The etiology of tumors is usually complex and many factors contributing to tumorigenesis and growth remain unknown. There is evidence for a variety of causes in mammalian tumors and there is no reason to assume that fish tumors differ in this respect. Known and suspected factors contributing to tumor formation in fish include viruses, chemical or biological toxins, physical agents, hormones, age, sex, genetic predisposition, and

host immunological competence. A genetic double can be closely linked to geographic location, which in turn can facilitate transmission of an infectious agent or promote the effects of a cancer-causing chemical. Carcinogens stimulate mutations in specific genes that become co-genes that are only active in certain mutant forms; many different carcinogens can cause the same mutation.

A viral and chemical etiology of tumors in wild fish has been suggested (Russell and Kotin, 1957). Today, epidermal neoplasms are known to be one of the most common tumors in fish (Harshbarger and Clark, 1990; Harshbarger and Slatick, 2001). The cause of papillomatosis appears to be most likely viral in some species, although the viral causative agent varies from species to species. No viral etiology has been found in some species, although in other cases papillomas appear to be more affected by chemical contaminants (Harshbarger and Clark, 1990). Epidermal papillomatosis is not usually fatal in adult fish. In addition, epidermal papillomatosis is easy and inexpensive to study in most fish species. For these reasons, epidermal papillomatosis could serve as a biological indicator of environmental stressors for many fish species where contamination and other environmental stressors are known to promote papillomatosis (Vethaake et al., 1992 Baumann et al., 1996). Typically, these previous field studies compared the prevalence of papillomatosis in populations sampled from contaminated and more pristine reference sites (Baumann et al., 1996).

1.9 HISTOPATHOLOGY

Histopathological examination is generally used to assess the manifestation of diseases and the health of organisms, as well as the structure of organs, reflecting the morphological structure of cells and tissues (Yevich and Barszcz, 1983). The tumor diagnosis is mainly based on the histological pattern (FenoglioPreiser et al., 2002). Similarly, neoplasms of lower animals are also classified after higher animals (Groff, 2004). Fish tumors are also classified based on the histogenesis and tissue origin of the neoplasm and whether the neoplasm is benign or malignant, although the histological characterization of a neoplasm as benign or malignant can be difficult to determine. Despite this, benign neoplasms are usually well-differentiated neoplasms that show a slow growth rate and have been described as a constant extensive mass often bounded by a fibrous capsule, while malignant neoplasms usually show faster growth than benign neoplasms and have variable cell differentiation, which may be well differentiated or undifferentiated (Cotran et al., 1999). Lack of differentiation of malignant neoplasm is often due to cellular anaplasia, characterized by pleomorphism or heterogeneity of neoplastic cells,

and cellular dysplasia, characterized by loss of uniformity and normal polarity or orientation of neoplastic cells. In tumors that are more anaplastic, the cells are difficult to identify and it becomes more complicated when a combination of tissues occurs.

Tumors of epithelial and mesenchymal tissue, whether internal or external surfaces or deep margins or glandular tissue, are characterized by an inability to grow into clumps or layers of similar cells, which can be used to indicate an epithelial or mesenchymal origin in even the most anaplastic cases. of neoplasms. They are also notable for their ability to stimulate the production of local proliferating capillaries and a supportive connective tissue stroma.

1.10 TUMOR IMMUNOLOGY

The tumor microenvironment includes tumor cells, extracellular matrix, immune cells, cytokines, and other factors that play important roles in tumor formation, growth, invasion, and metastasis (Liotta and Kohn, 2001). However, chronic inflammation is a driving force in the tumor microenvironment (Coussens and Werb, 2002), underscoring the importance of an effective immune response in controlling tumor initiation, promotion and progression (Coussens and Werb, 2001; 2002; Schreiber et al., 2011). Immune cells, particularly mast cells, lymphocytes, macrophages and null cells serve as a regulatory factor in the tumor microenvironment (Huang et al., 2008). Therefore, mast cell infiltration into the tumor may eventually remodel the tumor microenvironment and profoundly affect tumor behavior (Coussens and Werb, 2001; 2002). However, some studies have shown that mast cells promote tumor angiogenesis and tumor growth. Tumor-infiltrating mononuclear cells (MNCs) suppress various immune and cytotoxic activities against malignant cells (Miescher et al., 1986). The degree of mononuclear cell infiltration plays an important role in cancer prognosis (Svennevig et al., 1984). The tumor microenvironment associated with mast cells and mononuclear cells promotes or inhibits tumor cell proliferation, angiogenesis, and metastasis in higher vertebrates.

1.11 OCCURANCE PREVALENCE OF TUMORS IN MARINE FISH

Tumor cells are infiltrating, aggressive, invasive, and sometimes metastatic (Martineau and Ferguson, 2006). Neoplasms in lower animals are difficult to define precisely. The occurrence of sporadic and frequent tumors in cultured and natural fish populations has been reported in almost all tissue systems and has been widely reported by scientists and naturalists (Wellings, 1969; Mawdesley Thomas, 1971; Harshbarger, 1977). Field studies on natural fish populations

indicate that the occurrence and prevalence of tumors are dependent on fish size and sex (Korkea-aho et al., 2009). The prevalence of tumors is an indicator of environmental stress (Baumann et al., 1995). Tumors, such as epidermal neoplasms, are frequently observed in many fish (Harshbarger and Clark, 1990). Epidermal tumors can be seen as reddish to brownish white and black, fleshy to bony, sometimes loosely adherent to the skin and fins (Welling, 1969; Groff, 2004;

Gopalakrishnan et al., 2011). Internal tumors have also been found in almost all visceral organs such as liver (Cormier, 1986), pancreas (Fournie et al., 1988), ovaries (Romanucci et al., 2016), testicles (Borucinska et al., 2003), intestines (Magi et al., 2008), swim bladder (Bowser et al., 2012), thyroid (Marsh and Vonwiller, 1916), thymus (Kasantikul et al., 2015) and gills (Knusel et al., 2007). However, due to their peculiar appearance and obvious pathological character, more than 50% of fish tumors are associated with the skin (Mawdesley-Thomas, 1971; Anders and Yoshimizu, 1994).

A higher prevalence of tumors in fish has been reported in several parts of the world (Hussein and Mills, 1982; Yamamoto et al., 1985; Bullock and Minkoff, 1986; Smith et al., 1989; Moore et al., 1996, Pinkney et al., 2014). The prevalence of epidermal neoplasms in wild fish populations has been used as an indicator of environmental stress in both freshwater habitats (Hayes et al., 1990; Premdas et al., 1995; Baumann et al., 1995) and among seafarers (Vethaak et al., 1996; Mellergaard and Nielsen, 1995; Bucke et al., 1996). The causes of fish tumors are diverse and are often suspected to be multifactorial (Baumann 1992; Martineau and Ferguson 2006). Most of these tumors are of unknown etiology (Harshbarger, 1977). However, more than 50% of the causes of fish tumors have been suggested or proven to be due to infectious viruses (Yamamoto et al., 1976; Anders and Yoshimizu, 1994). The strongest evidence for the chemical etiology exists for polynuclear aromatic hydrocarbons (PAHs) in sediments, which have been implicated in the development of epidermal and hepatic carcinogenesis (Baumann et al., 1991; Malins et al., 1987 Vogelbeine et al., 1990). A causal relationship between PAHs and liver tumors or preneoplastic lesions in fish has been established by experimental studies (Schiewe et al., 1991; Metcalfe et al., 1988).

Seasonal variations in the occurrence and prevalence of skin tumors are well documented in several fish species (Harsbarger and Clark, 1990; Getchell et al., 1998). This is because seasonal variations can be affected by variations in water temperature, pH, dissolved oxygen and salinity. Wild European eel showed a higher incidence of neoplasms in summer when water

temperature was higher (Peters and Peters, 1977). Furthermore, the papilloma grew at 15 and 22°C but did not grow or even regressed under experimental conditions at 8°C (Peters and Peters, 1977). Some studies suggest that the seasonal cycle of endocrine activity may play an important role in neoplasm induction (Lee and Whitfield, 1992; Anders and Yoshimizu, 1994; Kortet et al., 2002). It is likely that temperature and hormone patterns could be causative factors influencing the occurrence of a neoplasm. Endocrine control of spawning is mediated by environmental cues, including changes in ambient water temperature (Jobling, 1995). Tumors appear in fish at the time of spawning, sexual maturity or smoltification (Lee and Whitfield, 1992; Anders and Yoshimizu, 1994). These are all life stages of fish with endocrine changes. Tumors have been experimentally induced and enhanced by injection of 17-β-estradiol and testosterone into white suckers (Premdas et al., 2001). Kortet et al. (2003) found that tumor was associated with increased testosterone concentration in wild male cockroaches. Sex steroids are known to suppress immune defense mechanisms and spawning stress in fish (Pickering, 1987; Watanuki et al., 2002).

Several field studies have shown that epidermal neoplasia is observed more frequently in large fish (Smith et al., 1989; Lee and Whitfield, 1992; Poulet et al., 1994; Mikaelian et al., 2000; Kortet et al., 2002) as well older fish (Smith et al., 1989; Mellergaard and Nielsen, 1995, 1997). This may be partly due to the effect of sex hormones on papillomatosis (Premdas et al., 2001) and the rarity of papillomatosis in immature fish. Field studies have also shown that gender can affect susceptibility to epidermal neoplasms. The form of epidermal neoplasia is more common and severe in male cockroaches than in females (Kortet et al., 2002) because male cockroaches have higher cortisol hormone levels than female cockroaches and higher testosterone levels in diseased cockroaches than male cockroaches during spawning (Kortet et al., 2003; Vainikka et al., 2004).

1.12 DEVELOPMENT OF A TUMOR

The extensive tumor-like masses have been observed in commercially important marine fish such as Flathead rodtail (Platycephalus indicus (Linnaeus, 1758)), mullet (Mugil cephalus Linnaeus, 1758), black catfish (Arius jella Day, 1877), Indian mackerel (Rastrelliger kanagurta (Cuvier, 1816)), spotted catfish (Arius maculates (Thunberg, 1792)) and highfin mullet (Upeneus indicus Uiblein and Heemstra, 2010). Among them, S. longiceps was most commonly affected by tumors, followed by S. jello. Less commonly, the remains were R. sarba

(3 cases), P. indicus (2 cases), M. cephalus (2 cases), A. jella (1 case), R. kanagurta (1 case), A. maculates (1 case).) and U. indicus (1 case).

1.12.1 Tumor Prevalence

Prevalence of neoplasms in Sardinella longiceps landed at Parangipettai Landing Center see Table 1. A total of 1,60,994 were examined, of which 707 had tumors, with an overall prevalence of 0.44%. The maximum prevalence per year (0.46%) was recorded in 2015, while the minimum prevalence (0.41%) was recorded in 2014. The maximum prevalence per season (0.81%) was recorded in the summer of 2015, while the minimum prevalence (0.14%) was recorded in 2014 after the monsoon was observed. Monthly, the maximum occurrence of tumors was recorded from June to August in 2014 and 2015. However, the highest tumor prevalence (1.14%) was observed in June 2014 (Fig. 35). The majority of tumors localized on the external region (99.26%) than the internal organs (0.74%). Within the external tumor, the majority was on the head (57.81%), followed by the fins (16.08%) and the body (7.88%) (Table 3). Among the internal organs, the stomach is often affected by tumors, while gonadal tumors are rarely seen.

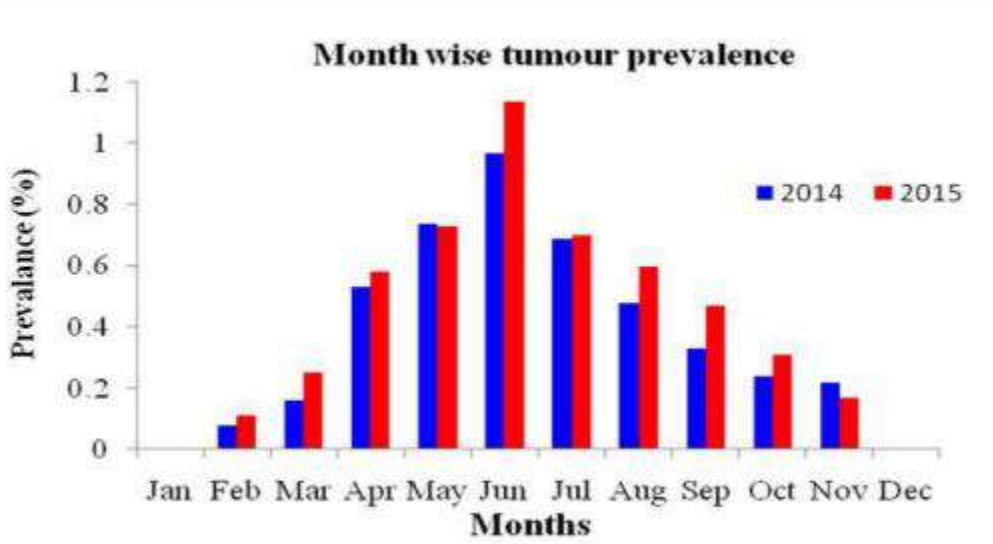


figure 3. Tumor prevalence in S. longiceps from the Parangipattai landing center

Table 1 Distribution of neoplasms to different areas of the *Sardinella body*

longiceps

Season	Distribution (%)						
	Head	Fins	Body	Head & fins	Head & body	Fins & body	Internal organs
Post-monsoon	51.20	17.91	8.16	14.24	5.58	2.43	0.48
Summer	58.87	16.91	7.79	11.92	2.34	1.44	0.73
Pre-monsoon	62.76	14.54	7.19	10.96	3.08	0.69	0.78
Monsoon	58.41	14.96	8.38	11.37	4.50	1.42	0.96
Grand total	**57.81**	**16.08**	**7.88**	**12.12**	**3.88**	**1.49**	**0.74**

1.12.2 Tumor Intensity

The highest intensity of sardine tumors recorded in a single fish was 76 scales. The seasonal mean intensity (±) of the disease ranged from 13.2 ± 0.9 to 27.8 ± 1.4 scales observed in the post-monsoon and summer, respectively (Table 4), with the average of the mean intensities during the season was 17.84 ± 6.13 scales. The prevalence of the examined neoplasms was between 0.14 and 0.81% (Table 1), the mean seasonal intensity of the neoplasms correlated significantly with the seasonal prevalence of the disease (R = 1.0, P = 0.896; Fig. 36).

Table 2. Mean tumor intensity in *Sardinella longiceps*

Seasons	Intensity		
	2014	2015	Overall
Post-monsoon	13.7 ± 0.7	14.2 ± 0.9	13.95 ± 0.8
Summer	16.1 ± 1.7	16.4 ± 2.3	16.25 ± 2.0
Pre-monsoon	27.4 ± 2.1	27.8 ± 1.4	27.60 ± 1.75
Monsoon	13.9 ± 0.8	13.2 ± 0.9	13.55 ± 0.85
Total	**17.77 ± 6.02**	**17.9 ± 6.23**	**17.84 ± 6.13**

1.12.3 Length to weight ratio

The length of the normal fish ranged from 14.0 to 19.9 cm and the weight from 28.0 to 83.0 g. The *b value* of pooled normal fish showed *b* =2.36 negative allomatric growth. *The b* - value of male and female normal fish showed *b* = 2.43 (Fig. 37A) and *b* = 2.27 (Fig. 37B) negative allometric growth, females showed *lower b* - values than male fish. The length of the tumor-infected fish varied between 16.9 and 20.5 cm and their weight between 30 and 90 g. The *b* value of pooled tumor-affected fish shows (*b* =1.7) negative allomatric growth. The *b value* of male and female fish with tumor showed *b* = 1.65 (Fig. 37C) and *b* = 1.41 (Fig. 37D) negative allometric growth, the female showed a *lower b value than* the male fish.

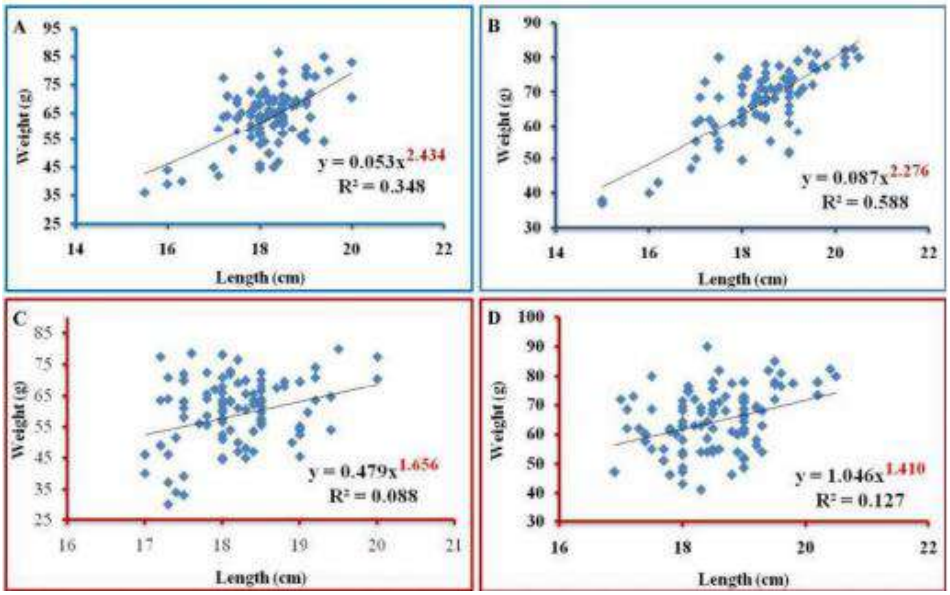


figure 4. Length and weight ratio of *Sardinella longiceps.* Normal; woman

(A)and male (B).Tumor affected; male (C) and female (D).

1.12.4 Immunopathology and etiology of fish tumors

The fundamental role of the immune system is to maintain tissue homeostasis through relentless immune surveillance and the initiation of inflammatory responses that involve coordinated activation of the innate and adaptive immune systems (Demaria et al., 2010). Inflammation is a positive response that activates against tissue damage and pathogens. Inflammation is an uncontrolled and chronic response that induces deleterious cellular transformation in the surrounding tissue (Hakansson et al., 1997). Neoplastic transformation alters tissue and cell structures, which induces immune responses and eliminates developing tumor cells. However, elimination is incomplete and the neoplastic transformation of the cells can evade immune control. Chronic inflammation is a driving force of the tumor microenvironment (Coussens and Werb, 2002), underscoring the importance of an effective immune response in controlling tumor initiation, promotion and progression (Coussens and Werb, 2001; 2002; Schreiber et al., 2011). The tumor microenvironment includes tumor cells, extracellular matrix, immune cells, cytokines, and other factors that play important roles in tumor formation, growth, invasion, and metastasis (Liotta and Kohn, 2001). Immune cells, including lymphocytes, macrophages, neutrophils, basophils, eosinophils, mast cells, and null cells, especially mast cells, serve as the regulatory factor in the tumor microenvironment (Huang et al., 2008). Therefore, mast cell infiltration into the tumor may eventually remodel

the tumor microenvironment and profoundly affect tumor behavior (Coussens and Werb, 2001; 2002). However, some studies indicate that mast cells promote angiogenesis and the growth of tumors.

Mast cells (MC) are bone marrow-derived, tissue-homing leukocytes implicated in the pathogenesis of harmful, even fatal, and allergic diseases. The infiltration and activation of mast cells in tumors was mainly mediated by tumor-derived stem cell factor (SCF). At low concentrations, SCF effectively induces chemotactic migration of mast cells (Okayama and Kawakami, 2006). Mast cells play an important role in the development of allergies and anaphylaxis, wound healing, angiogenesis and against pathogens (Huang et al., 2013). Because these mast cells contain large numbers of cytoplasmic granules of histamine, cytokines and heparin.

Tumor-infiltrating mononuclear cells (MNCs) suppress various immune and cytotoxic activities against malignant cells (Miescher et al., 1986). The degree of mononuclear cell infiltration plays an important role in cancer prognosis (Svennevig et al., 1984). Higher number of lymphocytes in tumor tissues than in normal tissues. These lymphocytes are mainly found in or near the stroma and not near the tumor cells (Allen and Hogg, 1985). Overall, 80% of the lymphocytes expressed T-cell properties and the rest were B-cells and null cells (Elbert, 1989).

The causes of fish tumors are diverse and are often suspected to be multifactorial (Baumann 1992; Martineau and Ferguson 2006). Most of these tumors are of unknown etiology (Harshbarger, 1977). However, more than 50% of fish tumors have been suggested or proven to be due to infectious viruses (Yamamoto et al., 1976; Anders and Yoshimizu, 1994). Virus tumors in fish have been reported based on TEM images of the size, shape and morphological structure of virus particles. In general, the most suspected viral particles present in fish tumors are members of the herpesvirus, adenovirus, papovavirus, and retroviruses, as well as unidentified particles.

1.12.5 Histopathological and histochemical diagnosis of fish tumors

The lack of differentiation in malignant neoplasms is often due to cellular anaplasia, characterized by pleomorphism or heterogeneity, and cellular dysplasia, characterized by loss of unity and normal polarity or orientation. Malignant neoplasms may also be characterized by increased mitotic activity, suggesting increased proliferation of neoplastic parenchymal cells, although this feature, if present, is not a hallmark of malignancy. Finally, the biological

behavior of malignant neoplasms leads to progressive infiltration and destruction of adjacent tissues, often accompanied by metastases characteristic of malignant neoplasms. Cancer is a general term used to describe a malignant neoplastic condition.

Microscopy is commonly used to study the anatomy of cells and tissues of living organisms. The ability to visualize and differentially identify microscopic structures often improves with histological staining. Histology is the study of the microscopic structures of cells and tissues, a discipline that has grown in importance since the development of the microscope in the 1500s and 1600s, making it possible to see specific structures in greater detail. Histology is an essential tool in clinical biology and pathology. Histological staining is widely used in pathology because it is easy to perform and its economic cost is relatively low. It also allows for the identification of multiple cell types and provides valuable information on cell structures, tissue morphology, and architectural changes associated with specific diseases (Yevich and Barszcz, 1983). Microscopic examination of diseased tissues and accurate diagnosis of cancer and other diseases generally require histopathological studies.

Dyeing is the artificial coloring of a substance to make it easier to study through the use of a colored organic molecule called dyes. The process can be technically progressive or regressive. In the former, the dye only interacts with the tissue until the correct staining is achieved, while in the latter, the tissue is overstained and the excess dye is then removed. On the other hand, to identify more than one cellular component in a sample; coloring can be accomplished by simultaneous or sequential addition of dyes that selectively color certain structures. This means that double, triple and multiple staining can be carried out. Specific intracellular and extracellular elements are visualized at the microscopic level using dyes that can react with defined tissue components

Hematoxylin-eosin (H&E) is the most commonly used dye to identify the nucleus or cytoplasm in cells. Hematoxylin is one of the most representative basic stains, capable of staining acidic structures of cell nucleus due to its DNA, RNA-containing organelles, ribosomes and rough endoplasmic reticulum. Eosin is the routine dye used as a counterstain in H and E staining. This is an acidic dye that can bind to ionized cationic groups on proteins such as the β-amino group of lysine and the guanidine groups of arginine. The Argyrophilic Nuclear Organizer Region (AgNOR) is one of the most common one-step silver staining methods that stains ribosomal RNA and protein synthesis. Toludine blue (TB) is the most common basic stain staining cytoplasmic heparin and histamine granules. Alicia blue is one of the cationic

dyes capable of staining ionized acid groups of phosphate groups of nucleic acids and carboxylate groups of glycoconjugates and proteins. Periodic acid Schiff (PAS) stained based on the presence of an adjacent pair of hydroxyl groups in sugars that can be oxidized with periodic acid, generating two aldehyde groups. Then these aldehyde groups are detected by the chromogenic Schiff's reagent. PAS strongly stains glycogen and some types of mucins, and is also useful for identifying basement membranes due to the sugar moieties of its proteoglycans. Sudan Black and Oil Red O are the most common one-step lipid stains that can identify lipid accumulation. Masson's trichrome (MT) is the most common multistep trichrome stain, staining the nucleus, cytoplasm, and collagen fiber. Van Gieson is the most common multistep trichrome stain, staining the nucleus, cytoplasm, and elastic fibers. Azo dye coupling is an organic reaction between diazonium and an aromatic compound that produces an azo compound that stains acid and alkaline phosphatase. Von Kossa and Alizarin Red S stains are the most common mineral staining methods that stain limescale. Von Kossa staining does not stain calcium, which stains and visualizes carbonate and phosphate moieties. Alizarin Red S is a chelating dye where the anionic dye binds to calcium. The aim of the present work was to investigate the occurrence of tumor types in fish, to present their histopathology and their special histochemical staining.

CHAPTER 2

THE ANATOMY AND PHYSIOLOGY OF THE TELEOSTES

Anatomy is one of the oldest disciplines in medicine, and the old saying goes "Anatomia medicinae fundamentum est". Therefore, by all standards one could have thought that the major lymphoid tissues in fish should have been discovered and described in 2000. But this was not the case. Since then, large previously unrecognized lymphoid structures have been identified in fish, and furthermore, ongoing research efforts have provided additional information on previously known tissues.

What is a lymphatic organ? An organ, whether lymphoid or not, can be defined as “a reasonably independent part of the body that performs a specific function or functions” (Studdert et al. 2012). Organs, in turn, are made up of tissues, and a good and useful definition of a tissue is “a group or layer of similarly specialized cells that together perform a specific function” (Studdert et al. 2012). Most tissues contain lymphoid cells, but that doesn't necessarily make them lymphoid. However, when the tissues contain mostly lymphoid cells, they are often organized into lymphoid organs. In other words, the exact definitions are not fixed. Following the terminology presented above, it is not easy to define a lymphoid tissue unless it can be visualized as an aggregate of lymphoid cells, such as mammalian Peyer's patches, which, although an integral part of the abdominal wall, are also defined are called secondary lymphoid organs. (Pabst et al. 2007). The reference to diffuse lymphoid tissue, common in pooled immunology, seems foreign to traditional anatomical terminology. In our view, the term "lymphatic tissue" outside of a well-defined localization composed primarily of lymphoid cells is confusing and should be avoided. For example, it is common to describe the lymphoid cells in the gut of fish as gut-associated lymphoid tissue, and that fish have "diffuse lymphoid tissue" there. Having a "diffuse tissue" contradicts the given definition of a tissue. Intestinal lymphocytes are part of a tissue (the intestinal mucosa), but do not form their own tissue. A more accurate terminology describing the presence of leukocytes in the gut of fish would be to refer to what is objectively observable, namely an infiltrated gut mucosa with mostly scattered leukocytes. The mucosa is divided into two constituent compartments, namely the epithelium and the lamina propria, which are separated by the basement membrane. The basement membrane is permeable and allows leukocytes to circulate from one compartment to another. This transport occurs through basement membrane windows, which are more prominent at sites with mucosa-associated lymphoid organs (MALTs) (Takeuchi and Gonda

2004). In mammalian anatomy, there is a clear distinction between the usual mucosal surface and that containing MALT structures that are only found in specific locations. As Smith et al. (2013) these places should not be confused. Therefore, although well established in the aquatic immunology community, we advocate not using terminology that implies that a “diffuse tissue” is a tissue. Hereinafter we use the term "lymphoid tissue" as an area where predominantly lymphoid cells are seen within defensive structural boundaries, and these sites are usually restricted to lymphoid organs. Therefore, this review does not address mucosa-associated lymphoid cells, which have recently been reviewed separately (Salinas and Miller 2015; Bjørgen et al. 2020), but excludes discrete lymphoid structures.

In our opinion, morphological studies of the fish immune system seem to have been somewhat forgotten in the age of genomics and proteomics. Zebrafish researchers tend to think of the fish as a swimming human, or at best a swimming human embryo with no biological rights per se. But teleost fish evolved millions of years ago (Zapata and Amemiya 2000) and are perfectly adapted to their way of life and habitat. They are not simply frozen at a certain stage of development in their evolution, but have developed their own solutions over the years. This fact often seems to have been overlooked by research that only focuses on fish as model organisms. A striking example of the discrepancy is the debate, or even lack of debate, about the existence of a system of lymphatic vessels in fish. It has long been known and proven that fish have a secondary vascular system. This secondary vasculature may appear as a lymphatic vessel, but it is not. However, some researchers seem to ignore this fact and have frequently published information about fish lymphatics. This is especially true for researchers using zebrafish as a model organism. Apparently her swimming human doesn't have a solution of her own. This neglect of available information prompted Vogel (2010) to publish an excellent commentary on the subject. In a notable comment he stated: “Given that a secondary vasculature has been established in all extensively studied actinopterygic fish; It would be extremely surprising if zebrafish were the only exception. Other researchers took this information into account when presenting their results, arguing for the existence of lymphatic vessels in teleosts (Hellberg et al. 2013). And that can definitely be the case. Based on the available information, it is reasonable to assume that bony fish have a secondary vascular system that is in no way equivalent to a system of lymphatic vessels, but that fish may also have lymphatic vessels that are most likely connected to the intestine (Hellberg et al. 2013). This is just one example of many where the scarcity of anatomical information has a major

impact on our perception of how the immune system works and where additional morphological studies are highly warranted.

Anatomy of the immune structures and organs of bony fish

2.1 THE INTEGUMENTARY SYSTEM

The skin is the main barrier against the environment and allows for normal internal physiological function, so its condition is important in many disease processes. The skin layers of teleosts, including the cuticle, epidermis, basement membrane, dermis, and hypodermis, are shown schematically in Figure 2.1.

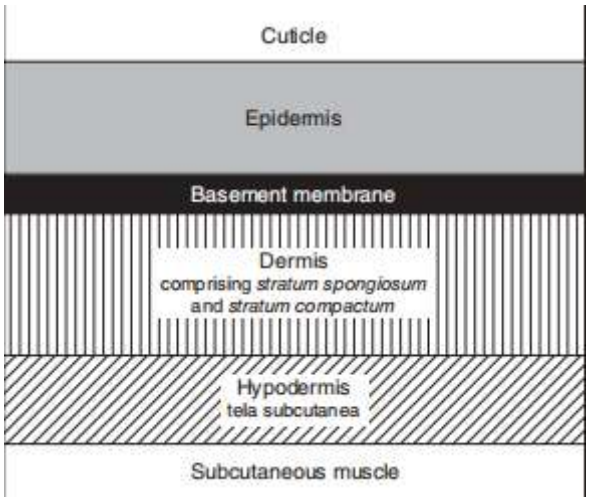


Figure 2.1 Schematic representations of normal teleost skin layers.

2.2 cuticles

The outer layer, the cuticle or glycocalyx, was first described in detail by Whitear (1970) as a predominantly mucosal polysaccharide layer with a thickness of about 1 μm. It is normally formed largely from surface epithelial cells rather than by the secretion of goblet mucosal cells, and is a complex of cell protoplasm, desquamated cells, and any goblet cell mucus secreted onto the surface (Figure 2.2). The physical consistency of the cuticle varies greatly between species and is particularly developed in rock pools and benthic species. The cuticle layer contains specific immunoglobulins and lysozyme (although the amount of the latter varies widely from species to species) and free fatty acids. As part of the skin's mucosal defense system, they are believed to have antipathogenic activity that works in conjunction with cell proliferation kinetics to continuously scavenge microorganisms from the surface (Speare &

Mirasalimi 1992). However, low bacterial counts are still typically found on these surfaces and there are obvious limitations to the effectiveness of such systems where there is a high environmental exposure to pathogens.

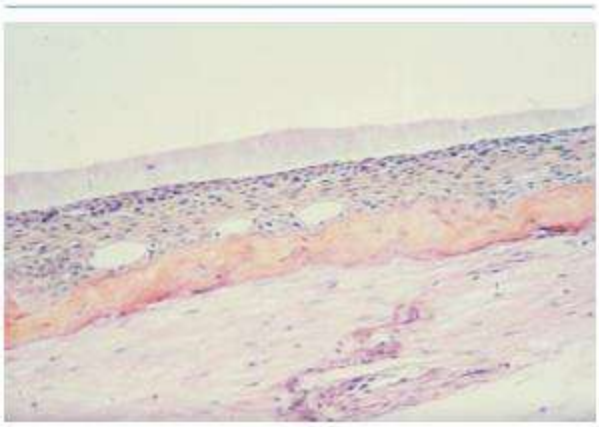


Figure 2.2 Whiting tail skin with cuticle, epidermis and dermis. PAS ×142.

2.3 EPIDERMIS As in all species of vertebrates, the basic unit of the epidermis of teleosts is the fibrous Malpighian cell. However, this is the only consistent feature as there is great diversity in all other cell types found there (Bullock & Roberts 1974). In adults, the epidermis is a stratified squamous epithelium covering the body surface and covering the tail and fins. Unlike its mammalian counterpart, it is alive and capable of mitodividing at all levels, even in the outermost layer of squamous epithelium. The surface of the outermost layer is arranged in a swirling pattern of micro-ribs (Figure 2.3).

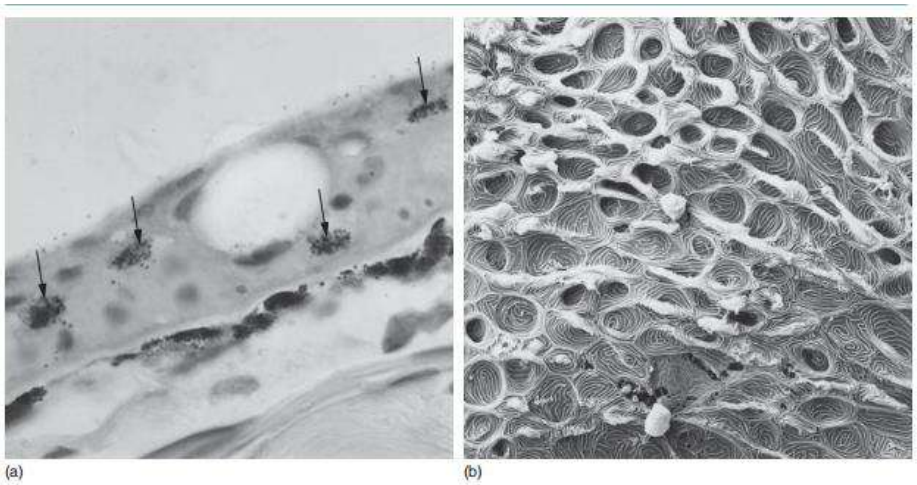


Figure 2.3 (a) Autoradiograph of a section of floe skin from a juvenile fish inoculated 12 hours previously with tritiated thymidine. The specifically labeled cell nuclei of dividing

cells (arrows) are found at all levels of the epidermis. H+E × 500. (b) Scanning electron micrograph of the guppy epidermal surface showing the characteristic arrangement of microridges. ×2200. (B courtesy of Dr. DK Cone.)

The thickness of the epidermis varies with species, age, location, and often the stage of the reproductive cycle. It is generally thicker in species with negligible scale coverage (e.g. eel) and also in finned species, where it is particularly well endowed with nerve end organs and mucous cells (Fig. 2.4).

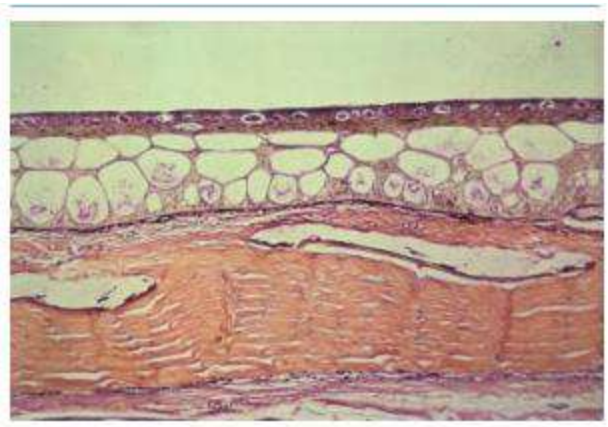


Figure 2.4 Section through the skin of a whiting showing a thick epidermis, with characteristic large cyst-like structures below and denser mucous epidermal tissue above. The following section also shows the scales and fissured skin tissue. PAS ×150.

Squamous cells are always present in the epidermis of teleosts. They are rounded cells, very similar in structure to the extreme levels where they are horizontally attenuated, with a cytoplasm consisting largely of a collection of elongated vesicles, degenerating mitochondria, and a few dense fibrous bundles, rather than the more typical widely distributed ones Bundles of fibers and mitochondria around a generally ovoid nucleus. Slime-secreting cells are found in the epidermis of all bony fish, but their number varies greatly by location and species.

These goblet cells usually arise from the middle layers of the epidermis, although a very thin epidermis will show a mucus cell having its base on the basement membrane. They increase in size and produce secretions (mainly glycoproteins) as they approach the surface. Mace cells are large, usually round cells found in the lower and middle layers of the epidermis of some groups of teleosts. The classic mace cells are the *shock substance cells in the epidermis of cyprinids that secrete a potent alarm substance, but many other species have large,*

morphologically similar, clear, non-mucous cells in their epidermis that do not appear to be related to such fearful reactions. Granule cells are found in a variety of teleost epidermis, but no function has yet been ascribed to them. Other cells found in the epidermis include lymphocytes, macrophages, and large, clear, cyst-like structures presumably of cellular origin, which are particularly prominent in Gadidae.

2.4 DERMIS

The dermis consists of two layers. The top layer, the *spongy layer,* is a loose network of collagen and reticulin fibers that is adjacent to the epidermal basement membrane. It contains the pigment cells (chromatophores), mast cells and scale bed cells, and the scales. The bottom layer, the *stratum compactum,* is the dense collagen matrix that provides structural strength to the skin. The ability to change color to match the environment or due to sexual activity or disease is highly developed in many teleosts and is induced by a controlled modulation of the interplay of absorption properties and reflection of chromatophores. Melanophores, dark pigment-containing cells, are asteroid cells that contain large numbers of electron-dense, membrane-bound melanin pigment granules that can be moved through the cell's cytoplasm to produce the desired effect. Lipophores are chromatophores containing organic solvents - soluble pigments - and are divided into erythrophores containing red pigments and xanthophores containing yellow pigments.

2.5 HYPODERMIS

The hypodermis is a looser adipose tissue that is more vascular than the overlying compact layer of the dermis and is a common site for the development of infectious processes.

2.6 THE MUSCULOSKELETAL SYSTEM

The spindle-shaped shape of the typical fish is determined by the demands of swimming. The streamlined external resistance of mini-putts and large muscle blocks (myomeres) are placed on either side of the axial skeleton to flex the body laterally and generate propulsive forces by swinging the body and tail.

2.7 AXIAL SKELETON

The layout of the skeleton can be seen in Figures 2.6, 2.7 and 2.8. The skull consists of a rigid skull to which the jawbones and the gill and opercular apparatus are articulated. The structure

is very complex and a large part of the skull moves during eating and breathing movements. All components are interconnected, and the structure of the skull is best understood in the context of a description of respiratory movements such as that of Ballintijn and Hughes (1965).

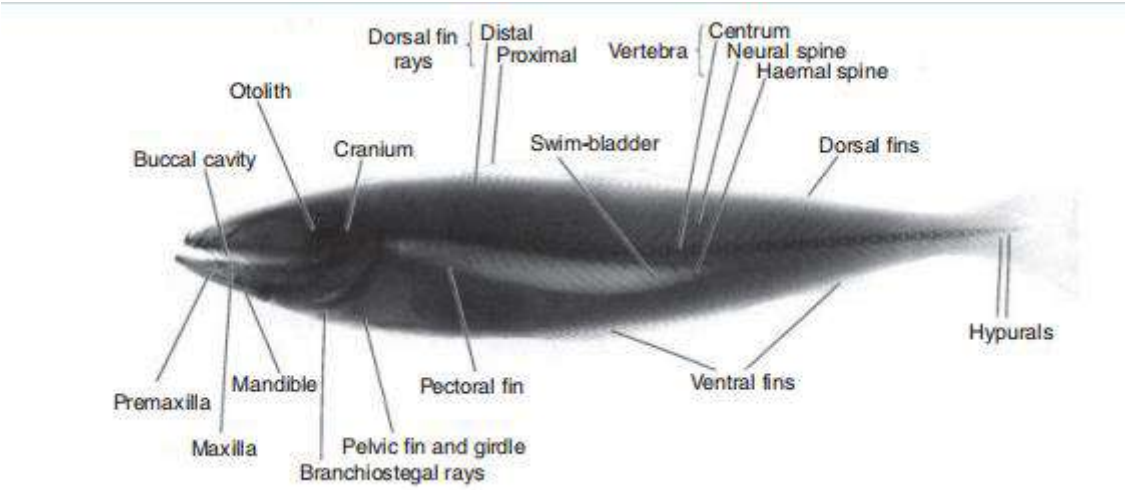


Figure 2.6 X-ray of a typical round fish, Pollock.

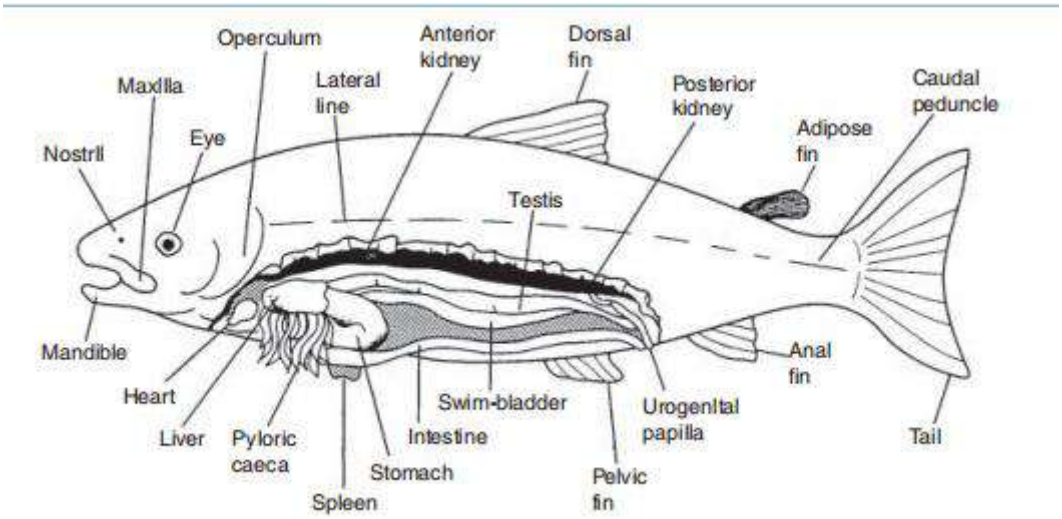


Figure 2.7 Diagram of the basic anatomy of a salmonid.

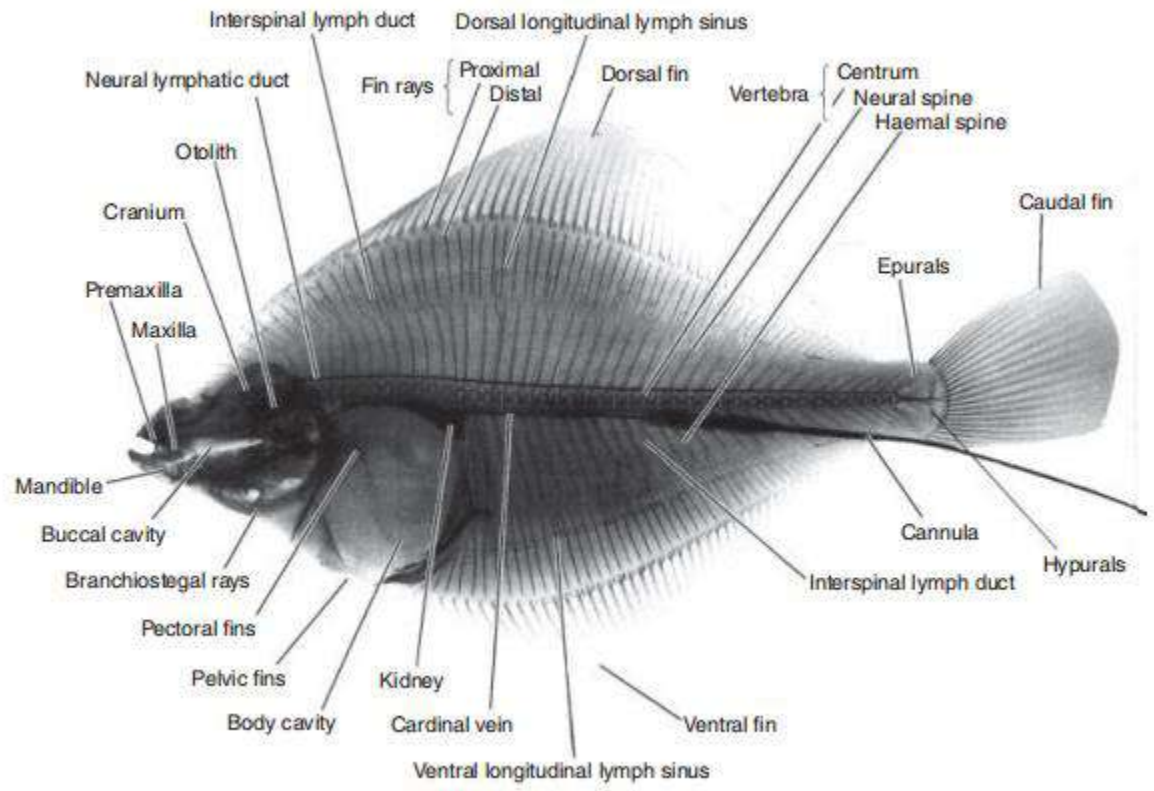


Figure 2.8 X-ray of a typical flatfish, plaice.

The neural lymphatic duct and renal portal vein were delineated by injecting a radiopaque substance through a cannula. The number of vertebrae is not constant within a given species and is influenced by environmental conditions during larval development. Each vertebral center is a simple cylinder, with the "cross" visible on X-rays reflecting the conical cavities surrounding the intervertebral pad. The edges of adjacent centers are connected by ligaments, and the entire column is held together by elastic longitudinal ligaments that pass dorsal and ventral to the vertebrae. All vertebrae have a neural arch and neural spine, with the caudal vertebrae also having a ventral hemal arch and hemal spine. In the thoracic region, instead of the hemal arch, there are pleural ribs that support the side walls of the body cavity. In many species, intermuscular bones of various configurations also emanate from the spinal column in the septa between the myotomes.

FINS

The pelvic girdle in lower bony fish (e.g. salmonids) is integrated with the ventral musculature of the body. In more advanced types, it is in a more forward position, resting against the pectoral girdle. The pectoral girdle hangs immediately behind the opercular region of the skull. The dorsal and ventral medial fins are connected to the pterygiophoral muscles, which extend

the line of neural and hemal spines. The caudal fifi n articulates on a series of flat plates, the dorsal epural and ventral hypural bones. The five rays can be of two types: prickly or soft. The five-rayed structure of bony fishes was once used to divide them into two broad groups: the Malacopterygii (corresponding roughly to the Isospondylii of current systematics), or soft-rayed species, and the Acanthoptergyii (corresponding roughly to current perchaceous species). The spiny rays are simple simple bones as in the first dorsal fin of the perciformes. The caudal rays of the fins of all teleosts are of the soft type, as are all other fins of the Isospondylii (e.g. salmonids and clupeids). The soft rays are segmented, often branching, and consist of two identical lateral components on either side of the midline. While the spiny Fifi n spokes are rigid, the soft Fifi n spokes are able to flex due to the activity of the muscles at the base of each Fifi n spoke pulling on the ligaments that run along the bony column. In this way, subtle movements are possible. The fine mesh of wild fish is clear and very fine, but it is often thicker in farmed fish.

BONE

The microscopic structural elements of fish bones are similar to those of other vertebrates, and two types of bone are generally found, cellular and acellular. The former contains osteocytes and is restricted to lower orders such as Clupeidae, Salmonidae, and Cyprinidae (Figure 2.9). Cellless bone is unique among vertebrates; it contains no osteocytes and is found in advanced teleosts such as Percidae and Centrarchidae, which often have a structureless solid matrix (Moss 1965). It has been shown that a lack of cells prevents the resorption of calcium from bone, so that acellular bone cannot function as a calcium store. The repair of fractures under calcemic conditions in advanced teleosts is therefore hampered (Moss 1965). Despite the presence of vascular canals and "medullary" spaces in some bones of the two main types, no hematopoietic tissue is present in these spaces. It is evident that bony fishes differ greatly in bone structure and physiology from other vertebrates.

MUSCLE

Most fish swim by passing a wave of increasing amplitude backwards along the body. This is most evident in the movement of eels (anguilliform movement), where the wave is generated by the sequential contraction of muscle blocks or myomeres from head to tail. In the more typical shorter-bodied fish, the mechanism is the same, but during swimming, the body movement shows less than a full wave and only the rocking of the tail is really visible

(carangiform locomotion). Some fish swim in a skull or wavy from certain fins, in this case the corresponding muscles are highly developed, and the large myomeres can be significantly reduced (e.g. hippocampus).

2.8 THE RESPIRATORY SYSTEM

The surface area of the gill epithelium is comparable to the total surface area of the skin and considerably larger in many species, making its structure important in the homeostasis of the fish 's *internal environment.* The epithelium is thin to allow for gas exchange, which also makes it particularly vulnerable to invasion by pathogens. In addition to the respiratory function, the gills are responsible for regulating the salt-water exchange and play an important role in the elimination of nitrogenous waste. Even slight structural damage can therefore leave a fish very susceptible to osmoregulation difficulties as well as breathing difficulties.

THE STRUCTURE OF THE BRILLIES

The gills of a typical bony fish consist of two sets of four holobranchs that form the sides of the pharynx (Figure 2.13). Each holobranch consists of two half-branchs projecting from the posterior edge of the branchial arch or gill arch in such a way that the free edges diverge and touch those of adjacent holobranchs. Careful examination of the hemibranches of a fresh gill shows that they consist of a series of long fivefold thin lamellae, the primary lamellae, which protrude from the arch like the teeth of a comb. The area of each primary lamella is further increased by the formation of regular crescent-shaped folds on its dorsal and ventral surfaces - the secondary lamellae. The dorsal and ventral rows of secondary lamellae on each primary lamella are staggered to complete the spaces in the rows of adjacent filamentation lamellae (Figure 2.14). This arrangement of arches and lamellae shapes the sides of the pharynx into two sets of corrugated screens through which water must flow.

VENTILATION AND GAS EXCHANGE

During respiration, water enters through the mouth, flows through the gills, and exits through the opercula. Respiratory flow is driven by alternate expansion and contraction of the buccal and opercular chambers, which act to maintain a continuous flow of water across the gills. Compared to air-breathing animals, the energy expenditure of gill ventilation is very high, especially when the water oxygen content is low, e.g. B. when it is hot or dirty. This is best demonstrated in aquaculture with respiratory distress syndrome, which occurs when the energy

required for gill ventilation exceeds the energy released by the extracted oxygen. Carbon dioxide is very soluble in water, so there is little difficulty in releasing it from the gills. In many bony fish, the breathing pump is stopped during swimming once sufficient speed is reached to allow ventilation of the gills, simply by opening the mouth and passing current across it. Known as "Dynamic Jet Ventilation", this system offers significant energy savings, and in fact fish like the largest tuna can only breathe by this method. Proprioceptors and mechanoreceptors that respond to changes in gill water flow are present to control ventilation. If, for example, the flow of gill water is artificially stopped, a reflex arrest of the heart occurs. Additionally, mechanical and chemical stimulation of the gills can trigger the cough reflex, thereby reversing the flow of water through the gill arches. The frequency of coughing has been found to be related to the level of irritating pollution. There are chemoreceptors on the gills and under environmental conditions of low P O 2 and high P CO 2 gill ventilation and increased heart rate. Aeration shows the greatest response, with more than 10-fold increases in fish exposed to severe hypoxia (less than 20% air saturation levels). An important receptor site in this context is the pseudobranch, a rudimentary gill located below the operculum, dorsal to the main gill arches. It is perfused by oxygenated blood from the first branchial arch and probably monitors arterial P O 2, but is also sensitive to hydrostatic pressure, Na + ions, osmotic pressure, pH and P CO 2. The pseudobranch is innervated by a branch of the glossopharyngeal nerve (ninth cranial nerve) and has other nonsensory functions, such as B. the hyperoxygenation of the choroid of the eye.

2.9 THE CIRCULATORY SYSTEM

The general structure of the circulatory system of a typical bony fish is shown in Figure 2.18.

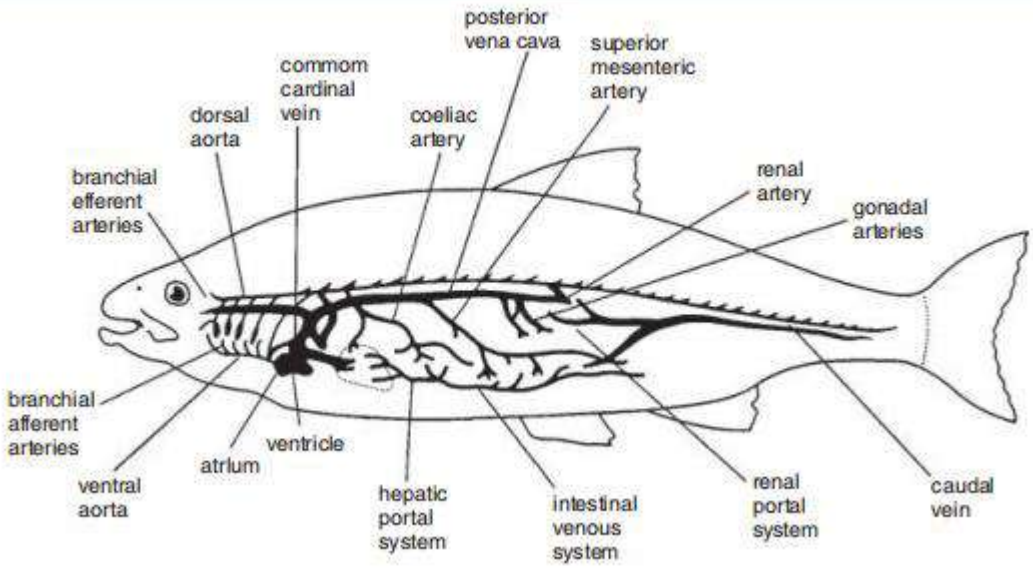


Figure 2.18 Schematic representation of the circulation of a typical bony fish.

THE HEART

The heart of teleosts is located in the pericardium in front of the main body cavity and usually ventral to the pharynx. It has four chambers through which blood flows in a simple sequence (Fig. 2.19). Venous blood depleted of oxygen enters the venous sinus from the canal cuvieri and the main veins. There are no inlet valves and the sinus is so small that it is difficult to identify as an inconspicuous ventricle. The wall is thin and composed mostly of collagenous connective tissue, although in some species it is muscular and contractile. In the wall of the venous sinus is the pacemaker that triggers the heart to contract. Blood enters the atrium, which lies dorsal to the ventricle, through two sinus-atrial valves. The auricle has a thin wall and trabeculae run through the lumen in a loose network. The endothelial mucosa therefore has a large surface area and, in some species, has phagocytic activity as part of the reticuloendothelial system. The contraction of the atrium forces blood through the valves into the ventricle. The ventricle has a much thicker wall than the atrium and only a minimal lumen is evident in normal histological sections. There is a distinct compact outer muscular layer and a spongy inner layer with numerous trabeculae. The thickness of the compact layer is related to the area of activity, as it is almost absent in less active species such as pleuronectids. The coronary vessels run around the outside of the ventricle and supply the compact muscle, with the spongy muscle getting most of its oxygen supply from the "venous" blood in the lumen. Even highly active fish, such as tuna, can have a coronary supply to the spongy layer (Tota 1989). Individual cardiac muscle fibers are about 6 μm in diameter, about half the diameter of mammalian

muscles. Otherwise, the fibers resemble those of mammals, with disks interspersed between each cell. From the ventricle, the blood is directed through two valves into the arterial piston. The globe has a thick wall composed of a mixture of elastic tissue and smooth muscle (Priede 1976). It has a complex structure but essentially acts as a passive elastic reservoir, smoothing the pressure pulse from the ventricle and maintaining blood flow during ventricular diastole. The elastic tissue of the globe has a completely different structure than the elastic tissue of the arteries.

ARTERIES

The ventral aorta arises from the heart and distributes blood to the gills via the branchial arteries that feed it. The arteries supplying the gills have a normal vertebrate arterial structure with three layers in the wall: external adventitia, media, and intima. The endothelium consists of flfl attenuated cells, which can usually only be distinguished by their dark stained nucleus, which protrudes into the lumen. Adjacent cells interlock so that the endothelium forms a continuous surface. There is a thin basement membrane beneath the endothelium, but this is only visible under an electron microscope. The intima is mostly elastic tissue and the media consists of lamellae of elastic tissue interspersed with smooth muscle cells. The wall of the ventral aorta is very elastic and can contract to accommodate blood flow (Kirby & Burnstock 1969). The outer adventitia is thin and composed mainly of collagen fibers. The efferent branchial arteries join to the dorsal aorta dorsally of the pharynx, with the exact pattern varying by species. Blood flows from the first efferent gill through the pseudobranch and from there to the eyes and skull. In this region, too, the arteries branch ventral to the pharynx to supply the lingual and coronary systems.

VEINS

The veins of fish, like those of other vertebrates, are relatively inextensible and have walls composed primarily of collagen. The main veins are large in diameter and the pressures are low, less than 10 mmHg (1.5 kPa), although there is no evidence of negative pressures like those experienced in sharks. There is renal portal drainage through the kidneys, mainly from the caudal region, and from the intestines there is a hepatic portal system typical of vertebrates. Valves are not common in the teleost vein system.

TRAFFIC CONTROL

Cardiac output can vary widely, with changes in stroke volume being more pronounced than changes in heart rate. The heart has inhibitory vagal innervation and adrenergic stimulating innervation has also been demonstrated in some species (Gannon & Burnstock 1969). The heart also responds positively to increased venous return according to Starling's law. The general increase in blood flow during exercise can be explained by the effect of circulating catecholamines on alpha-receptors in different parts of the body. Vasomotor nerves have also been demonstrated, and while it is evident that there are many interspecific differences in bony fish, many mechanisms known to mammalian physiologists have their counterparts in bony fish.

CAPILLARY

In mammals, capillary (hydrostatic) blood pressure opposes an equal blood colloid osmotic pressure across the capillary wall. This seems impossible in fish due to the low blood pressure, but the capillaries are very permeable, so the osmotic pressure across the wall is much lower than in other vertebrates (Hargens *et al.* 1974). The interstitial fluid has a high protein concentration and in fact the plasma circulates fairly freely through the capillary walls. The fluid balance in bony fish differs fundamentally from that in mammals, so that, for example, fairly large fluctuations in plasma concentration can be tolerated without any problems.

LYMPH

The lymphatic drainage system of fish is very extensive, probably due to high capillary permeability. The lymphatic volume is approximately four times the blood volume (Wardle 1971) and its composition is almost identical to that of blood plasma. In most fibroids, the only available circuit is the lymphatic system because there are no significant blood vessels in white muscle. Various lymphatic propellants or "lymphatic hearts" are located along the large lymphatic vessels, which facilitate the return flow of lymph during respiratory movements (Kampmeier 1969). A unique feature of the circulatory system of fish is the presence of a secondary circuit in the form of narrow tortuous arterial vessels from the primary gill vessels and also the arterial supply of various surfaces such as the skin and intestines. Because the majority of blood cells are carried through the primary circulatory system to the tributaries of the two systems, blood in the secondary system usually has a lower hematocrit and pressure. Its circulation time may therefore be on the order of hours rather than the minutes required for primary circulation (Steffenson & Lomholt 1992; Iwama & Farrell 1998).

2.10 Hemoglobin and gas transport

Most teleosts, like other vertebrates, have hemoglobin in their erythrocytes. Because blood temperatures are often low, much oxygen can be carried in simple solution in the plasma, and so some arctic fish have no hemoglobin. There is considerable variation in fish hemoglobins, with up to four types occurring in an individual, each with its own characteristics. Also, many fish have different hemoglobin levels at different stages of development. Species can be adapted to different environmental oxygen tensions, and acclimatization to different temperatures results in altered oxygen dissociation properties. The Bohr effect, in which an increase in *PCO2* or a decrease in pH decreases oxygen affinity, occurs particularly in fish adapted to conditions of high oxygen and low carbon dioxide. Fish living in acidic, low-oxygen waters would not benefit from a Bohr shift. In many fish there is an additional phenomenon where low pH lowers the overall oxygen carrying capacity of hemoglobin and shifts the dissociation curve. This so-called root effect is unique to bony fish. It greatly facilitates the delivery of oxygen to tissues and is of great importance in high-pressure oxygen secretion in the gas gland of the swim bladder and in the choroid plexus of the eye. Total respiratory gas exchange at the gills and tissues is strongly influenced by the rate of oxygen loading and depletion in fish blood hemoglobin. In the gills, the pH is about 7.4, and here the rate of oxygenation is about four times faster than the rate of deoxidation. But at low pH, which is typical for actively metabolizing tissues, the process is reversed and the rate of deoxygenation is 400 times faster than oxygenation.

BLOOD COMPOSITION

The blood volume of teleosts is small compared to all other classes of vertebrates, on the order of 5% of body weight.

PLASMA

The composition (mg/100 ml) of fariotrout serum is given by Wolf (1963) as chloride 424, sodium 358, magnesium 2.3, potassium 20.1, calcium 12.5, phosphorus 9.3, sulfate 0.8 and whole blood glucose 71 specified; freezing point depression is about 0.57°C. This is remarkably similar to mammalian serum, and indeed mammalian saline solutions have been used successfully for fish tissue culture. Frog Ringer's solution is low in NaCl and KCl, hypotonic and not suitable for working with fish. Plasma protein concentrations are lower than in humans (7 g/L), with values ranging from 1.68 to 6.19 g/L being measured in various teleost

species. The immunological and other functions of the proteins are broadly similar to those of higher vertebrates, but many interspecific differences remain to be explored.

CELLULAR COMPONENTS OF BLOOD

The cellular components of fish blood differ from those of higher animals primarily in the nucleation of red blood cells and the presence of nucleated platelets. These are the source of prothrombin rather than the anucleated platelets of higher animals. There are also species differences with respect to specific elements, and the role of neutrophils and eosinophils is less obvious in teleosts.

2.11 HEMOPOIETIC TISSUE

Because teleosts lack lymph nodes and their bones generally lack a medullary cavity, the hematopoietic tissue is located in the stroma of the spleen and the interstitium of the kidney. It is also found to a lesser extent in the periportal areas of the liver, the intestinal submucosa, and the specialized lymphoid organ, the thymus.

KIDNEY HEMOPOIETIC TISSUE

In the kidney, hematopoietic tissue provides a supportive matrix for the nephrons of the posterior kidney, but the anterior or great kidney is almost exclusively hematopoietic. The blast cells reside in a stroma of reticuloendothelial tissue similar to that of mammalian bone marrow. Endothelial cells line many paranasal sinuses, through which blood from the renal portal vein flows to filter dead cells and add new ones. Stannius corpuscles and internal (adrenal) tissues (cortex and medulla) are embedded in hematopoietic tissue. Another cellular structure present throughout the hematopoietic tissue of teleosts but not in higher vertebrates is the melanomacrophagic center (Roberts 1975b). The melanomacrophage centers differ in their degree of organization depending on the species. Inferior teleosts are clusters of dark cells distributed throughout the hematopoietic tissue (Figure 2.25). The degree of melanization varies with age, but at any age the pigment present is dark brown or black and has all the biochemical and chemical properties of melanin, although not necessarily bound to the characteristic melanosomes of integumentary melanin (Roberts & Agius 2003). In higher teleosts, the amount of dark pigment in the melanomacrophage centers of the spleen or kidneys of normal fish is usually very small, with most of their pigment being much lighter (Figure 2.26). Histochemically it is lipofuscin, but gem (1971) provided evidence suggesting a very

close chemical affinity between lipofuscins, or aging pigments, and melanin. The morphology of the melanomacrophage centers of higher teleosts is also much better defined. They are usually nodular with a delicate argyrophilic capsule. In many species they fit closely to the vascular channels and may have a collar of lymphocytes. Circulating macrophages filled with particles, possibly of microbial origin, or metabolic waste products such as ceroid or hemosiderin selectively attach to rophage melanomac centers, which can therefore be considered as metabolic dumps.

THE SPLEEN

The spleen is the only lymph node-like organ found in bony fish. It is dark red or black in color and, when healthy, usually has sharply defined borders. It is located near the greater curvature of the stomach or intestines. Although usually single, in some species it may be divided into two or more smaller spleens. The splenic capsule is fibrous and devoid of muscle and lacks the dense trabeculae that extend into the tissue found in the mammalian spleen. In some species, the pancreas is located as the subcapsular layer of the spleen, but in most fish the main elements of the spleen are the ellipsoids, the pulp, and the melanomacrophagous centers. Ellipsoids are the thick-walled filter capillaries that form when the splenic arterioles divide. Each consists of a thick basement membrane - a contiguous tube in which the vessel extends, usually eccentrically, separated from the membrane by a layer of sheathed compartments. These contain erythrocytes and phagocytes and are capable of capturing large amounts of particles from the circulation.

THE THYMUS

The thymus is a paired organ, an ovoid pad of primary lymphoid tissue located subcutaneously in the dorsal commissure of the operculum. It arises from the primordia, which are connected to the epithelium of the pharyngeal pouches. Lele (1932) performed a comparative study of thymic morphology in bony fish and found that its lifespan varied greatly between species, coiling before sexual maturity in lower bony fish but surviving several years after maturity and even in higher bony fish growth.. In cross section, the thymus is an aggregate of small fibrous capsule lymphocytes and five argyrophilic supporting cells. Macrophages are very numerous in the thymus of some fish (e.g. *Lophius*) and are found in close association with lymphocytes. The thymus is believed to be the site of maturation of immunocompetent T cells. Occasionally,

epithelial strands are observed and, rarely, focal epithelial clusters, which may correspond to Hassl bodies.

2.12 The reticuloendothelial system

The reticuloendothelial system (RES) is the system of scavenger cells widely distributed throughout the body and responsible for removing dead cells and particles from the circulatory system. The criteria by which cells are incorporated into the RES is high phagocytic activity and the ability to concentrate and separate this phagocytosed material. The weight of evidence suggests that in mammals, and probably teleosts, tissue macrophages derive from blood-circulating monocytes and their progenitors; there are therefore two populations of macrophages, one fixed and the other motile. Teleost cells considered to be RES are promonocytes from hematopoietic organs, monocytes from blood and lymph, macrophages from loose connective tissue, free and fixed macrophages from the spleen and kidney, and in many species the adherent macrophages of the atrial mucosa of the heart. The most important organs are the kidneys because of their greed and the atrium because of its particularly vulnerable spot. There are a number of differences between the mammal and the bony fish when it comes to RES. The atrial mucosa is a very important site in many species of fish, while it does not show such activity in higher animals (Figure 2.28). There are no lymph nodes in fish, and the liver, whose Kuppfer cells provide the largest surface area of phagocytic tissue in mammals, is virtually inert to phagocytosis in teleosts (Ferguson *1975a*)

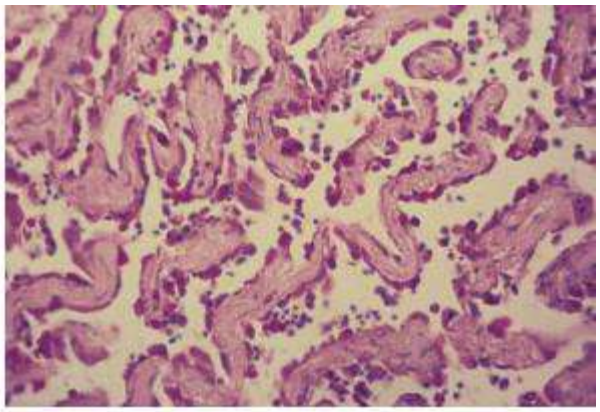


Figure 2.28 Section through the pinna of a plaice injected with yeast cells 6 h before.

The adhering scavenger cells of the atrium have absorbed the yeast and are turning bright red. NOT ×760. Giant cells and epitheloid cells found in chronic inflammatory lesions of teleosts are also considered part of the RES, or more specifically the "mononuclear macrophage

system" (Van Furth 1970) since they are formed by the fusion of individual macrophages in the presence of certain non-degradable irritants (Timur 1975). The anatomy of the different tissues that have a phagocytic component is described under the respective organ systems. An interesting feature of macrophages in the teleost RES is their ability, fixed or circulating, to form aggregates upon saturation. Commonly, these aggregates are found in the melanomacrophage areas of hematopoietic tissues (Roberts 1975b), but such aggregates are also found, often pigmented, in or around chronic inflammatory lesions. It is not known whether the pigments found in such aggregates are of purely exogenous origin, nor is it known why such aggregates are so frequently collared by lymphocytes. However, it is believed that melanin and related pigments play a defensive role in their ability to produce H_2O_2 in many organisms

2.13 THE RENAL AND EXCRETRIAL SYSTEM

Regulating the composition of the internal body fluids of fish is a complex process. Adult skin is essentially impermeable, but as discussed in the Osmotic and Ionic Regulation section of this chapter, water and ionic fluxes readily occur through the gills. Other surfaces where transmission occurs are the intestinal wall and the kidneys. It is therefore the regulation of flow in the three organs - gills, kidneys and alimentary tract - that accounts for the osmoregulatory and excretory needs of fish.

THE EXECUTERING KIDNEY

The bony fish kidney is a composite organ of hematopoietic, reticuloendothelial, endocrine, and excretory elements. The first three functions are all discussed elsewhere, and this section is limited to examining the excretory component. The kidney of bony fish is normally located retroperitoneally on the ventral side of the spine. It is a light or dark brown or black organ that usually runs the full length of the body cavity. It is generally divided into the anterior or main kidney, which consists primarily of hematopoietic elements, and the posterior or excretory kidney. Although it appears embryologically as a paired structure, its adult form varies between species, from two separate parallel organs in species like the anglerfish, to varying degrees of attachment, to the complete fusion found in salmonids. The ureters, or ureteral ducts, which carry urine from the collecting ducts to the urinary papilla, may fuse at any level and, after fusion, expand into a bladder (Fig. 2.29). The urinary tract opens to the outside behind the anus.

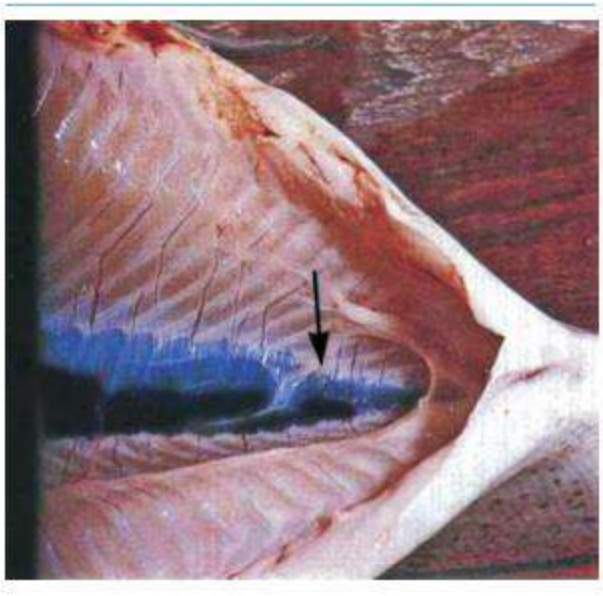


Figure 2.29 Rainbow trout kidney with white archinephric ducts joining to form the bladder (arrow).

Arterial blood is supplied to the kidney by the renal arteries coming directly from the aorta or segmental vessels. With the exception of the few agglomerular types, these serve the glomerular capillary bed and then open into the efferent arterioles. In marine and euryhaline species, the peritubular capillaries also receive blood from the caudal or segmented vessels, drain the caudal region, and form a renal portal system. So this blood is venous (Hickman & Trump 1969). The structure of the teleost nephron varies greatly between marine, euryhaline, and freshwater forms, reflecting the significant differences between their respective functions. The typical freshwater teleost nephron includes a well-vascularized glomerulus (Figure 2.30), a ciliated neck, two distinctive proximal segments (one with a prominent brush border and the second with abundant mitochondria but a less developed brush border), a narrow ciliated intermediate segment, a distal segment and a system of collecting ducts. The marine nephron is usually smaller and consists of a glomerulus, a cervical segment, two or three proximal segments forming the main component, an intermediate segment sometimes found between the first and second proximal segments, and the collecting duct system. The euryhaline teleost generally has a nephron that combines the structure of the two types and approximates that of the marine teleost, with the addition of a distal segment resembling that of the freshwater teleost. Some marine and freshwater species are abnormal in that they lack glomeruli in the nephron. There are many variations of individual species in the structure of nephrons, and the descriptions in this section provide only a general summary. The most important work in this

area is that of Hickman and Trump (1969), which should be consulted for more detailed comparative information.

OSMOTIC AND IONIC REGULATION

In fresh water, the environment is hypoosmotic and water tends to pass through the gills and permeable surfaces of the pharynx into body fluids. This is compensated by the kidneys, which produce large amounts of dilute urine, so glomerular filtration is of great importance. Ions are also lost passively through the gills and significant amounts are lost in the urine. The latter is minimized by urine with very low concentrations of Na+ and Cl-. The balance is maintained by active uptake of Na+ and Cl- by the gills and uptake of food through the intestinal wall. In the marine environment, blood has a relatively low osmotic pressure, so passive diffusion causes water to be lost through the gills and ions to be taken up. Urine is produced in small quantities with low or negligible glomerular filtration rates. Urine is approximately isosmotic or slightly hypoosmotic and contains mainly the divalent ions Mg^{2+} and SO_4^{2-}. Drinking removes large amounts of seawater to compensate for passive water loss. Divalent ions are generally not absorbed by the intestines and therefore appear in the stool. Excess monovalent ions in the body are excreted through the gills.

2.15 THE DIGESTIVE SYSTEM

THE DIGESTIVE TRACT

Bony fish feed on a very wide variety of substrates, so differences in oral structure and in the digestive tract, adaptations and specializations to adapt to specific diets are often very pronounced. The overall length of the digestive tract is a big difference, with that of herbivorous fish being much longer than that of carnivorous species. Other dietary specializations, including dentition, the presence and number of diverticula, and even the complete absence of a stomach in some species, are also of significant taxonomic importance (Figure 2.32). The swim bladder, embryologically derived from the upper digestive tract but without any digestive function, is considered a distinct system.

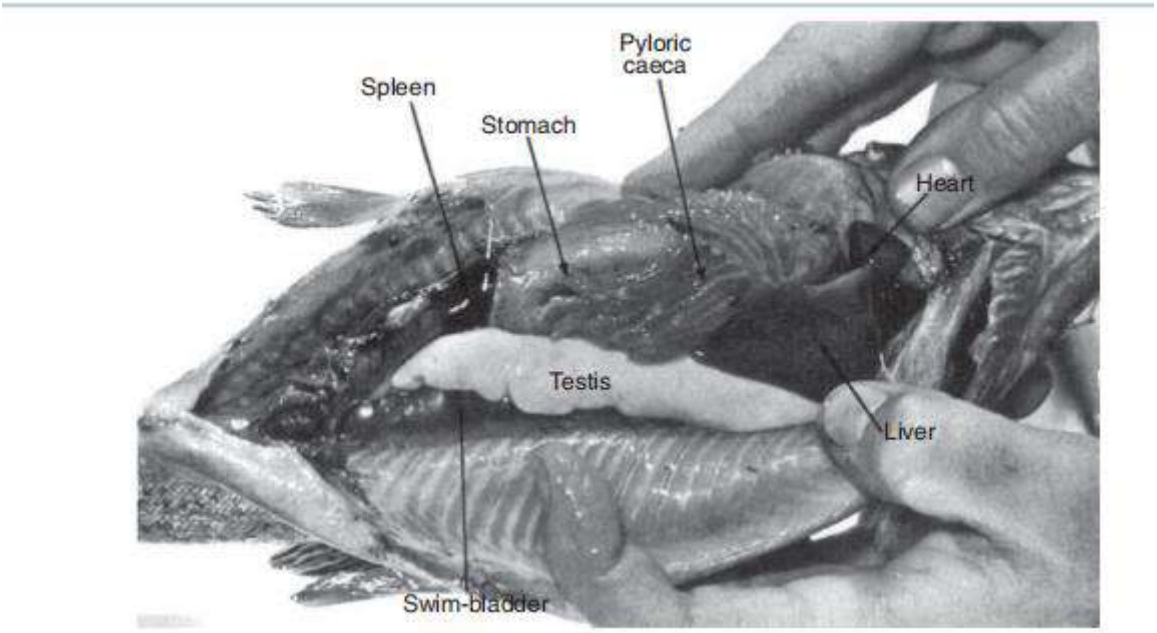


Figure 2.32 The abdominal contents of a rainbow trout, a top predatory carnivore with a very short gut.

LIVER

The liver of bony fish is a relatively large organ. In wild fish, it is usually reddish brown in carnivores and light brown in herbivores, but it can be yellow or even cream in color at certain times of the year. Farmed fish, whose diet generally contains higher levels of lipids, are usually lighter in color than their wild counterparts. The liver may be an organ located in the anterior abdomen, or in some species may have processes that lengthen the length of the abdomen or fit snugly to the other viscera. In some species it is a compound organ in the form of a hepatopancreas, in others the pancreas is a separate organ. Fish liver histology differs from that of mammals in that there is a much lower tendency for hepatocytes to be arranged in strands or lobules and the portal vein triads typical of mammalian liver are not visible. The sinusoids, irregularly distributed between the polygonal hepatocytes, are less numerous and lined with endothelial cells with very prominent nuclei. Functional Kuppfer cells are not found in the lining of the sinusoids. This was first demonstrated by Varichak in 1938 and has since been adequately confirmed (Ellis *et al.* 1976), but descriptions of so-called Kuppfer cells based exclusively on morphological criteria keep appearing (Hinton & Pool 1976). Sinusoidal lining cells are fenestrated and line Disse's space, which is the area between sinusoidal cells and hepatocytes and contains microvilli of both and numerous fat storage cells, Ito cells.

Hepatocytes are polygonal and have a characteristic central nucleus with densely stained chromatin borders and a prominent nucleolus

THE PANCREAS

Pancreatic tissue is more variable in location, even within the same species, than other abdominal viscera. The most common sites are scattered islands of secretory tissue interspersed with fat. The cells are found in the mesentery of the pyloric cecum, as the subcapsular lining of the spleen, and as the outer layer around the portal vein of the liver. The acinar structure of exocrine pancreatic tissue is very similar to that of mammals and consists of cells with very dark basophilic cytoplasm. When actively feeding on fish, they contain large numbers of shiny, eosinophilic secretory granules. The pancreatic duct normally empties into the common bile duct somewhere along its length. The endocrine components of the pancreas, the islets of Langerhans, are a series of lightly encapsulated, lightly stained structures composed of small spindle-shaped α, β, and δ cells. The size of the islet cells can vary seasonally, and in some species there is a large islet known as Brockman's body.

NUTRITION, METABOLISM AND GROWTH

Most knowledge of the nutritional needs of fish is based on work on artificially cultured salmon and trout. More recently, however, information on the dietary needs of farmed omnivorous and herbivorous species such as carp and tilapia has become available for comparison purposes (Jauncey 1982, 1999). The main energy foods are carbohydrates, proteins and fats, as in all other vertebrates.

carbohydrates

Although many fish species can use both carbohydrates and lipids as a source of energy, carbohydrates are not generally a major dietary component in amounts up to 25% of the diet. Essentially, the energy metabolism of fish is similar to that of a diabetic mammal. Therefore, ingestion of glucose for several hours will produce persistent hyperglycemia. This slow glucose metabolism is partly due to low catabolic activity in tissues such as liver, muscle and kidney. Insulin derived from fish lowers blood sugar and promotes transmission to tissues, but it appears that insulin is not needed to modulate blood sugar in bony fish. While in mammals rapid glucose homeostasis is necessary to maintain brain function, in fish higher glycogen stores in the brain can make this response unnecessary.

PROTEIN

The protein requirements of fish are related to the growth and development of the gonads. In fact, for a variety of fish, there is a clear linear relationship between daily protein requirements and specific growth. This implies that protein utilization is relatively constant and independent of diet category (i.e. carnivore, omnivore and herbivore). The dietary protein requirement of fish is between 45% and 70% of the gross energy value of the diet. This seems high compared to cultured homeotherms. On the other hand, the total energy required to maintain a constantly elevated body temperature for a homeotherm is greater than that of a similarly sized ectotherm. This difference therefore leads to a higher proportion of non-protein energy components in the diet. When daily protein requirements are related to meat production, all fish categories have a range of 420-766 g/kg live weight gain, which is no different from that of farmed land animals.

LIPIDS

Fish need lipids for energy production and to maintain the structure and function of cell membranes. At lower body temperatures than homeotherms, fish use low melting point lipids. Many commercially important marine species feed on crustacean zooplankton, which contains high levels of polyunsaturated fatty acids in the form of waxy esters. As a result of this diet, fish such as herring and capelin deposit large amounts of fat in the liver and muscles. Constituent lipids are oils composed of triglycerides containing ω 3 -series polyunsaturated fatty acids. Having such large amounts of oils, accounting for 10-20% of body weight, means that lipids and not carbohydrates are the main store of energy. As a general rule, body fat content is inversely proportional to body water content; it is a useful index of the condition of the fish under certain circumstances. In general, increasing the non-protein components of the diet results in better protein utilization. Diets containing between 10 and 20% fat by weight optimize protein utilization, although commercial salmonid diets today often contain up to 30%. In addition, the protein-saving effect of lipids is more effective than that of carbohydrates. However, excess lipids can lead to mortality associated with fatty liver, particularly in salmonids. It has been shown that polyunsaturated fatty acids of the ω 3 series are essential components of the fish diet. Deficiency can lead to growth arrest, caudal fin erosion, fatty liver, cardiac myopathy, and the development of shock syndrome. When linolenic acid (24:6 ω 3) is added to the feed at levels of about 1% by weight or 2.7% of the total energy content, growth and improved feed conversion are stimulated. Bell (1998) gives a comprehensive overview of the lipid nutrition of fish. Freshwater species achieve the highest weight gains on diets

containing both ω 3 and ω 6 fatty acids. This last series is more typical of terrestrial organisms and may reflect the origin of the diet in freshwater fish. Interestingly, the dietary needs of a given fish species closely match the body composition of this wild-caught species, the latter presumably feeding on an all-natural diet. There are, as expected, non-energetic dietary requirements for minerals and vitamins. These requirements are broadly similar to those of higher animals, and there is a wealth of information gradually accumulating about the effects of their deficiency in the diet.

METABOLIC RATE

Measuring metabolic rate, primarily in the form of oxygen consumption, provides estimates of the energy needs of fish, both for rearing and for water management. In addition, as aquatic heat cycling, fish are very sensitive to changes in their environment, which is reflected in changes in metabolic rate. Therefore, measuring oxygen consumption is a sensitive technique for determining the relative importance of different environmental factors. Because of the variability in metabolic measurements, standard turnover is taken as an arbitrary non-activity level extrapolated from the relationship between swimming speed and oxygen consumption, measured after at least 24 hours of fasting.

2.16 THE REPRODUCTIVE SYSTEM

Bony fish exhibit greater diversity in their reproductive patterns than any other group in the animal kingdom. Although most species have both male and female sex, both maphroditism and bisexuality occur. Parthenogenesis (development from an unfertilized ovum) and gynogenesis (development from an ovum stimulated to divide by invasion of a sperm not contributing genes) are also recorded, either in nature or in the laboratory (Purdom 1972). Eggs and sperm may be released into the water for external fertilization, or copulation may occur, resulting in either the release of fertilized eggs or the viviparous release of fry (Hoar, 1969). Young fish may be hatched in nests and guarded by a male or female, hatched from eggs laid in reeds, released from eggs floating in plankton, or even held in a parent's mouth. An understanding of the reproductive anatomy and physiology of teleosts and their pathophysiology is particularly important in farmed species, where egg production and larval stages are usually most critical in terms of economic efficiency and the biology of the system (Bromage & Roberts 1995).

The testicles

The testes are paired organs suspended by mesenteries on the dorsal abdominal wall adjacent to or below the swim bladder. Their size varies from small strands of tissue in juveniles to large, flat, white abdominal organs that make up about 12% of total body weight. There is a main collection duct for genital secretions from the testicles - the vas deferens - which carries mature sperm to an excretory duct at the urinary papilla. The testicle itself consists of a series of blind tubules or sacs, the seminiferous tubules, lined with spermatogenic (or seminal) epithelium. The process of male gamete maturation involves the proliferation of spermatogonia, or sperm progenitor cells, which develop from the spermatogenic epithelium to form spermatocytes. Many of them eventually undergo meiotic division to become haploid sperm. Sperm attach to the surface of semiiniferous epithelial pyriform feeder cells called Sertoli cells until they are ready to be released. Testosterone, the male secondary sex hormone, is secreted by the testes' interstitial cells, which are located in the fibrous supportive tissue or basement membrane of the seminiferous tubules.

The ovary

The structure of the female genitalia of bony fish varies from the simple collection of ovarian follicles found in the lower bony fish to the highly complex organs found in viviparous species. This not only produces eggs, but also acts as a sperm store, vagina, and uterus where young embryos can be nurtured. Mature ovaries can account for up to 70% of total body weight. They are suspended from the abdominal wall by a mesentery and usually appear as small clusters of tiny orange-white balls in immature fish. The primary ovarian cells are the ovarian follicles. These line a cavity or potential cavity that has a very complex sequence of folds in its coating. Eggs are passed through this cavity as they mature. In higher teleosts, eggs are transferred directly through an oviduct, but more primitive species such as salmon nests transfer eggs through a fold in the mesentery, which eventually ruptures, releasing the eggs directly into the abdominal cavity, for example. Evacuation through the genital opening. The oogonia, the cells that begin to mature, are surrounded by a single layer of small epithelial cells, and this collection of oocytes and epithelial cells is known as the ovarian follicle. Epithelial cells develop as the egg grows and are separated from it by a gradually thickening hyaline capsule, the zona pellucida. These granulosa cells, as they are called, are responsible for nourishing the egg and secreting its yolk. When an egg degenerates before ovulation, it invades the degenerating cell before being invaded by macrophages and melano-macrophages. In many species, several generations of ovules can be found at different stages of development

2.17 THE NERVOUS SYSTEM

The teleost nervous system extends throughout the body as an interconnected system of integrating centers and pathways: neurons and their axonal and dendritic processes. The greatest concentrations of nervous tissue are found in the brain and its posterior extension, the spinal cord, and these together make up the central nervous system (CNS). The peripheral nervous system (PNS) includes the nerves emerging from the CNS and their nerve endings or special sensory organs. Only part of nerve function is under conscious control—the nerves that serve the striated myotomal muscles and the brain voluntary muscle. The regulation of heart rate, chromatophores, respiratory movements of the gills, peristalsis, and other smooth muscle functions are controlled by autonomic components of the system, as in higher species. The neurons of fish are similar to those of other species, except that some, like those of the Mauthnerian groups, are very large compared to those of mammals. Supporting cells, neuroglia (astrocytes, oligo-godendrocytes and microglia), are also present. CNS tissue is divided into classic gray and white matter, consisting of nerve and neuroglial nuclei, or myelinated axonal processes (Ariëns Kappers *et al.* 1960). The brain and spinal cord are protected by a single primitive meningeal layer, the meninx primitiva, which contains the cerebrospinal fluid (CSF) produced by utilizing the choroid plexus. These ependymal, glomeruli-like invaginations of the ventricles are often found in very different locations in the brain compared to mammals, due to differences in the folding of the teleost brain. The roots of the spinal nerves, particularly in the region of the dorsal root ganglia, are normally covered with assemblages of eosinophilic granule cells morphologically similar to those commonly seen in the intestinal submucosa of teleosts and other loose connective tissues.

THE BRAIN

The teleost brain is similar in its basic components to the brain of higher animals, but has many differences in shape and complexity.

SPINAL CORD

The spinal cord of bony fish extends along the body and, in higher bony fish, terminates with an endocrine structure called the urophysis. The gray and white matter in the spinal cord of teleosts is well delineated and increases in complexity with evolutionary level, although the two gray matter dorsal horns are fused. They contain many large motor neurons in the posterior and anterior horn tissues. The dorsal and ventral roots of the spinal cord do not have a

demarcation of motor and sensory nerve fibers as in higher vertebrates: the two bundles contain a mixture of nerve fibers. The main features of the medulla are the very large ventromedial axons, the Mauthnerian axons of the medulla.

PERIFARY NERVES

There are 10 cranial nerves that serve both sensory and motor, voluntary and involuntary functions of the head and, in the case of the vagus nerve, also the parasympathetic supply of important visceral organs.

2.18 **NAGATIVE BLADDER**

STRUCTURE

The gas-filled swim bladder is a notable element (up to 7% of body volume) and characteristic of bony fish guts (Figure 2.47). Its main function is that of a buoyancy mechanism since the teleost body has specific gravities 107% and 105% of that of freshwater and seawater, respectively. It is also used for picking up sound and pressure, and in some species is equipped with drum muscles to produce sounds. The swim bladder is absent in many bottom-dwelling species where neutral buoyancy is not required, and in some fast-swimming pelagic species where it would increase drag by increasing surface area. In the fish larva, the swim bladder develops as a dorsal diverticulum from the foregut, so many structural features of the digestive tract are retained in the adult. Histologically, it consists of two main layers: an inner layer lining the gas space and an outer layer. The inner tunica has a transitional epithelial layer covering a muscular mucosa and a loose vascular connective tissue submucosa. The outer tunic consists of an outer serosa, beneath which is a tough fibrous layer that houses the muscles and elastic connective tissue. The embryonic connection between the gut and the swim bladder is preserved as a pneumatic conduit in many of the most primitive bony fish (Isospondyli). Fish with this type of swim bladder are called "physostomes". In most stingray fish, the functional pneumatic conduit is lost: the closed swim bladder or "physoclist". In physostomies and physoclistes, the swimbladder shows a wide range of morphological variations related to habitat and behavior (Figure 2.48). Many phytostomotic swim bladders have two chambers separated by a diaphragm. The anterior chamber is then associated with the uptake and retention of gases and therefore has a thicker wall. The posterior chamber, involved in the absorption of gases, has a fine inner tunic.

LIFT ADJUSTMENT

Neutral buoyancy depends on maintaining a constant volume in a flexible, gas-filled buoyancy chamber, regardless of the depth of the fish. In physostomies with access to the water-air interface, inflation is generated by swallowing air, which is then pushed through the pneumatic line to the swim bladder. In physoclists and physostomies without access to the water-air interface, inflation occurs through the release of arterial blood gases that pass through a gland located in the tunica interna of the anterior ventral portion of the swim bladder. Gas reabsorption occurs when a capillary plexus (the oval) of the dorsal aorta is exposed to gas from the swim bladder. The oval is an impermeable muscle membrane that controls the area of the exposed plexus and therefore absorption. The components of the gas are mainly oxygen, nitrogen and carbon dioxide, but the proportions differ from those of air in many cases. The partial pressure of oxygen can reach 176 atmospheres in some species of deep-sea makrurids. In physostomies such as cyprinids, the swim bladder contains pure nitrogen, while carbon dioxide is a more variable component found mainly in physoclists (for more information see Steen 1970; Tytler 1976).

RECEIVING SOUND AND PRESSURE

The perception of sound and pressure is similar in that the propagation of sound in water involves pressure oscillations and the speed and movement of water particles. Thus, the flexible, gas-filled swim bladder, which responds to changes in pressure by changing volume, is an obvious potential pressure receptor. The swim bladders of many species have a direct or indirect connection with the perilymphatic system of the inner ear. Although nearby sound sources can be sensed by fish without swimbladders by bone conduction, otolith vibration, or lateral line response, they are insensitive to distant sound sources above 400 Hz. With the inner ear (e.g., Gadidae), good conditioned responses can be generated at frequencies below 520 Hz, while clupeids, ictalurids or cyprinids with their direct connection can have perceptual frequency ranges between 13 and 4000 Hz. The presence of Weber ossicles depresses the high frequency response up to 5000 Hz. As with buoyancy, any uncompensated change in depth affects the sensitivity of sound perception by altering the volume of the swim bladder (Chapman & Hawkins 1973). Fish with a swim bladder can perceive relative pressure changes of less than 0.5% of the surrounding hydrostatic pressure, while fish without a swim bladder can only perceive pressure changes between 2.5% and 10% (Tytler & Blaxter 1973).

SOUND PRODUCTION

Although they lack a larynx, some fish can produce sounds by rubbing the jagged surfaces of specialized skeletal components. Many species in the families Sparidae, Tetrodontidae, and Holocentridae produce a high-pitched sound by grinding their teeth, but vibration of the swim bladder wall by specialized muscles provides the largest repertoire of sounds or calls produced in fish. Members of the Triglidae are known for their vocal production. Indeed, the twilight choruses of blackbirds caused great confusion among anti-submarine echolocation operators aboard United States Navy warships during World War II, until the target was finally identified (Moulton 1956).

2.19 THE ENDOCRINE SYSTEM

The endocrine system of fish shares the same basic components as that of higher vertebrates, but exhibits many distinctive features due to the vast differences in environmental stresses and evolutionary development that bony fish experience (Matty 1985). The most striking difference is the number of endocrine structures that have no apparent analogue in mammals. These include the urophysis, corpuscles of Stannius and possibly the pseudobranch.

The pituitary

As in all vertebrates, the teleost pituitary consists of two embryologically distinct components. These are the neurohypophysis, which develops from the diencephalon of the brain, and the adenohypophysis, which arises from an upward budding of the ectoderm of the embryonic oral cavity. The two fuse with their respective mesenchymal vasculature to form a compound endocrine gland surrounded by the diencephalon above and later joined by a bony cupula, the cella turcica, below. Extracts of pituitary tissue are often used to stimulate ovulation in farmed fish, and the protection these structures offer makes its removal from carcasses somewhat difficult. The bony fish neurohypophysis is much simpler than that of mammals and comprises a stalk of nerve tissue with a bulge at its end, forming the nucleus of the complete gland. The trunk consists of the axons of neurosecretory neurons whose cell bodies are located in the nuclei of the hypothalamus. The adenohypophysis secretes a variety of protein or peptide hormones and is generally anatomically divided into an intermediary and distal part. It consists of a number of different cell types with different dye properties. The exact correlation of different cell types with their specific hormones has not yet been fully elucidated and may differ from species to species (Ball & Baker 1969). Teleost pituitary hormones can be divided into two

groups: those that stimulate the activity of other endocrine organs (eg, thyroid, gonads, and adrenal glands) and those that affect physiological processes such as skin melanophore movements, osmoregulation, metabolism, and growth. A more detailed description of the teleost pituitary and its hormones can be found in the excellent review by Matty (1985).

THYROID

The thyroid gland of bony fish has a basic structure similar to that of mammals, and the thyroid hormone that stimulates many metabolic processes is an iodinated thyroxine, similar to that in higher animals (Gorbman 1969). Thyroid follicles, as in mammals, are generally round to oval with a low cuboidal epithelium and PAS-positive colloid secretion, but a very important distinguishing point is the anatomically diffuse distribution of the follicles, which varies considerably from species to species, even from one individual to another.. Instead of being localized in an inconspicuous capsule, they are distributed in the connective tissue of the pharyngeal region or even around the eye in some species, the ventral aorta, the hepatic veins, and the hematopoietic tissue of the kidney (Fig. 2.49). This diversity in distribution has led to a number of reports of neoplastic follicles, but although thyroid tumors do occur, most of these reports are probably just normal ectopic follicles. Thyroxine and triiodothyronine are controlled by pituitary thyroid-stimulating hormone (TSH). Both are transported in the blood bound to plasma proteins. Unlike in mammals, thyroid hormones in fish do not elicit a caloric response, but appear to affect carbohydrate metabolism and mobilization of lipid stores. These fish responses to thyroid hormones are highly dependent on nutritional status, ambient temperature, photoperiod and salinity. Recent work has shown that Roxin promotes growth in juvenile fish by stimulating appetite.

SURRENALS (INTERRENALS AND SUB RACE)

The compact kidney-like endocrine gland, which encompasses both cortex and medulla, is only present in a few groups of teleosts such as bullheads. Normally, in teleosts, an equivalent of the adrenal cortex, the intermediate gland, is a series of lightly stained cuboidal eosinophilic cell cords located in the anterior kidney, often in association with the large blood vessels that run through the region (Figure 2.50). The steroid hormones of the adrenal cortex include gluco- and mineralocorticoids, and these have functions very similar to those of higher animals. The adrenal medulla, the chromaffin tissue (so called because of its staining reaction to chromium salts), can be localized in various ways. It is found concomitant with the sympathetic ganglia,

in clusters between the anterior kidney and the spine, or, as mentioned above, in close contact with the interrenal tissues within the main kidney. It secretes sympathomimetic substances like adrenaline that are associated with immediate responses to stress. In particular, increased blood levels of catecholamines cause hyperglycemia and increase the functional area of the gills for gas and ion exchange.

ULTIMOBRANCHIC GLANDS

All vertebrates have the ability to regulate their serum calcium, and in fish this is accomplished through the activity of the ultimobranchial glands, strands of polygonal cells lying just ventral to the esophagus in the septum separating the venous sinus from the abdomen (Copp 1969). The organ is an embryonic fifth branchial arch and is functionally equivalent to the mammalian parathyroid gland.

2.20 STANNIUS BODIES

Stannius bodies are usually paired whitish accumulations of endocrine tissue, usually located retroperitoneally on the surface of the kidney. Large clear endocrine cells appear to secrete at the center of the cluster (Figure 2.51). They secrete a glycoprotein hormone called teleocalcin, which blocks the uptake of calcium by the gills, making them resemble the parathyroid glands of higher animals in some respects. They also have other properties such as secretion of pressor substances and possible involvement in osmoregulation, but they do not appear to secrete steroid compounds.

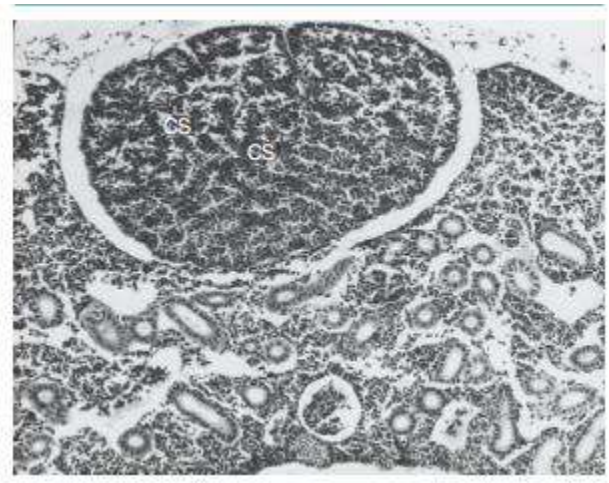


Fig. 2.51 Section through a corpuscle of Stannius (CS) of the median kidney of a rainbow trout. H+E ×35.

THE ENDOCRINE PANCREAS The distribution of pancreatic tissue varies considerably between species. The endocrine component is also diverse, with small islets of Langerhans scattered throughout the tissue in salmonids or eelfish. In higher teleosts, the endocrine tissue consists of a small number of scattered islets and one large, compact islet that varies in size with life cycle stage and is known as the Brockman body (Epple 1969). They have a delicate fifibrous capsule that contains the three types of islet cells, cells that produce glucagon, cells that produce insulin, and cells of unknown function. There are significant changes in islet size at spawning and senility and with dietary changes, but there are also reports of seasonal differences in the proportions of different cell types. Insulin causes hypoglycemia, but fish do not exhibit the rapid response to blood glucose clearance typical of mammals. Insulin release is linked to blood sugar levels, but only to allow relatively low levels to exit the extracellular space to act as cellular fuel. It may target oxidative glucose clearance but not glycogen deposition. Insulin has been shown to inhibit gluconeogenesis from amino acids and reduce hepatic protein turnover. Insulin's main role may be to conserve proteins and amino acids and to promote tissue deposition. Glucagon antagonizes insulin by increasing blood sugar through hepatic glycogenolysis. It also stimulates the incorporation of amino acids in the liver and stimulates gluconeogenesis.

UROPHYSIS

The caudal endocrine secretory structure is found only in sharks and bony fish. Its function is still somewhat unclear, but its anatomy has been well described by Bern (1969). It is a small whitish ventral extension of the spinal cord at its posterior end. Like the rest of the medulla, it is covered with a meninge and has a very extensive vascular supply that drains to the renal portal system. It consists largely of large neurosecretory cells with polymorphic nuclei whose myelinated axons extend from the medulla in a urophyseal stalk similar to that of the pituitary. They terminate in palisades adjacent to the capillary walls of the neurohemal complex, which are related to those of the hypothalamus. The urophyseal hormones are peptides primarily concerned with osmoregulation, although one acts specifically on the smooth muscle of the reproductive tract (Bern & Nishioka 1993).

THE Pseudobrantal and Choroidal Bodies

The pseudobranch is not present in all teleosts, but when present it is a red gill-like structure derived from the first gill arch and attached to the inner surface of the operculum. It consists of

parallel blood capillaries supported by cartilaginous rods. The pseudobranch has a direct vascular connection with the choroid of the eye, which consists of similar networks of capillaries alternating with rows of thin fifi broblast cells. Fish that do not have a pseudobranch (e.g. Anguillidae) invariably lack a choroid. Although it is believed to have endocrine and regulatory functions, as well as a hyperoxygenating function for the retinal vasculature, these have yet to be defined in detail.

gonads

In addition to their apparent gametogenic function, bony fish gonads also secrete hormones that have a general effect on a variety of tissues. Their production is controlled by the production of gonadotropins from the pituitary gland, the "conductor of the endocrine orchestra". Estrogens and androgens produced in the gonads cause skin thickening, color changes, the development of reproductive tubercles, kypes, and swelling of the urogenital region.

CHAPTER 3

TELEOST MUCOUS IMMUNITY

The mucosal surfaces of teleosts (bony fish) are the primary interface between fish and their immediate environment and serve as the primary entry points for most pathogens. The mucosal surfaces of fish include the epithelium and associated tissues of the gills, skin, gut, and reproductive tract. In mammals, the mucosal system consists of an integrated network of tissues with associated immune cells called the mucosa-associated lymphoid tissue **(MALT). It** is generally accepted that a comparable system exists in teleosts, although much less is known about its cellular and molecular components and the extent to which they function independently of the systemic immune response. Although a general understanding of the immune system of bony fish is emerging, fundamental questions remain regarding the development of primary lymphoid organs, the induction, enhancement and differentiation of local mucosal immune responses, the production of mucosal antibodies and lymphocyte effectors, and immune memory. Answers to these questions will lead to a better understanding of the evolution of basic immunological mechanisms as well as insights of direct interest for applied vaccines and the protection of farmed fish from microbial infections.

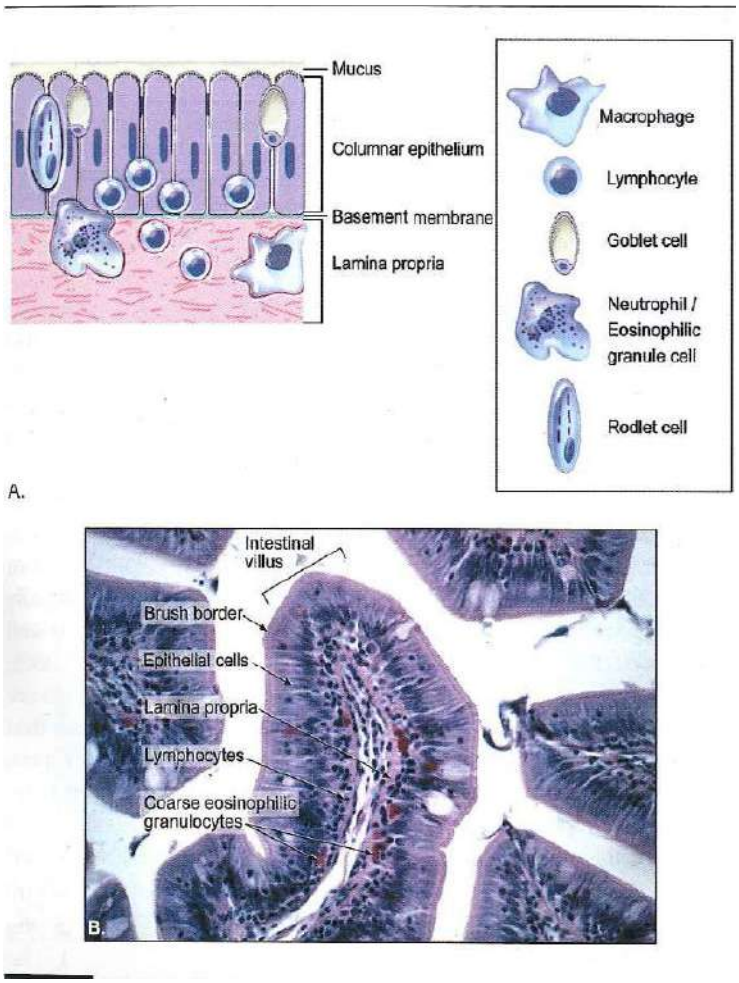
A number of laboratories have been or are currently investigating mucosal immunity in various fish including carp (Cyprinus carpio) (Rombout et *al.,* 1993), *channel catfish (Ictalurus punctatus)* (Lobb, 1987; Hebert et al., 2002), rainbow trout (Oncorhynchus *mykiss)* (Bromage, 2004), Atlantic salmon *(Salm° salar)* (Lin *et al.,* 1998), perch *(Dicentrarchus labrax)* (Picchietti *et al.,* 1997), zebrafish *(Danio* rerio) (Danilova and Steiner, 2002) and others Because bony fish are a diverse group of fish, understanding the biology of their immune system requires a comparative approach. A general understanding of mucosal immunity emerges from the synthesis of research from different laboratories on several species of fish, and these concepts are presented in each of the sections of the chapter.

3.1 ORGANIZATION OF MUCUS TISSUE AND RELATED IMMUNE CELLS

The respiratory and digestive systems share the mouth and oral cavity. The lining of the oral cavity consists of a mucoid epithelium layered over a thick basement membrane with a dermis connecting the epithelium to underlying bone or muscle tissue (Roberts, 2001). The esophagus has an epithelial lining with a large number of mucus cells. The size of the stomach

varies depending on the fish species examined. The gastric mucosa is mucous with many glands in the crypts of the mucosal folds (Roberts, 2001).

Although the intestinal morphology of bony fish varies by species and diet, the intestinal tract shares a common basic structure. The gut is a single tube without the anatomically distinct large intestine found in mammals (Roberts, 2001). The rectum has a thicker muscular wall than the intestine and is highly mucogenic (Roberts, 2001). The esophagus, stomach and intestines consist of four basic layers, which differ in their composition



A. Diagram of the basic anatomical structures of the intestinal epithelium and the identification and location of associated cells related to the immune system.

B. Photomicrograph of the intestinal villi of a channel catfish. Note the mucosal edge of the brush, tall columnar epithelial cells (enterocytes), and the supporting lamina propria containing migrating lymphocytes and coarse eosinophilic granulocytes (hematoxylin and eosin [H& Ej staining). between and within each of these organs. The innermost layer is

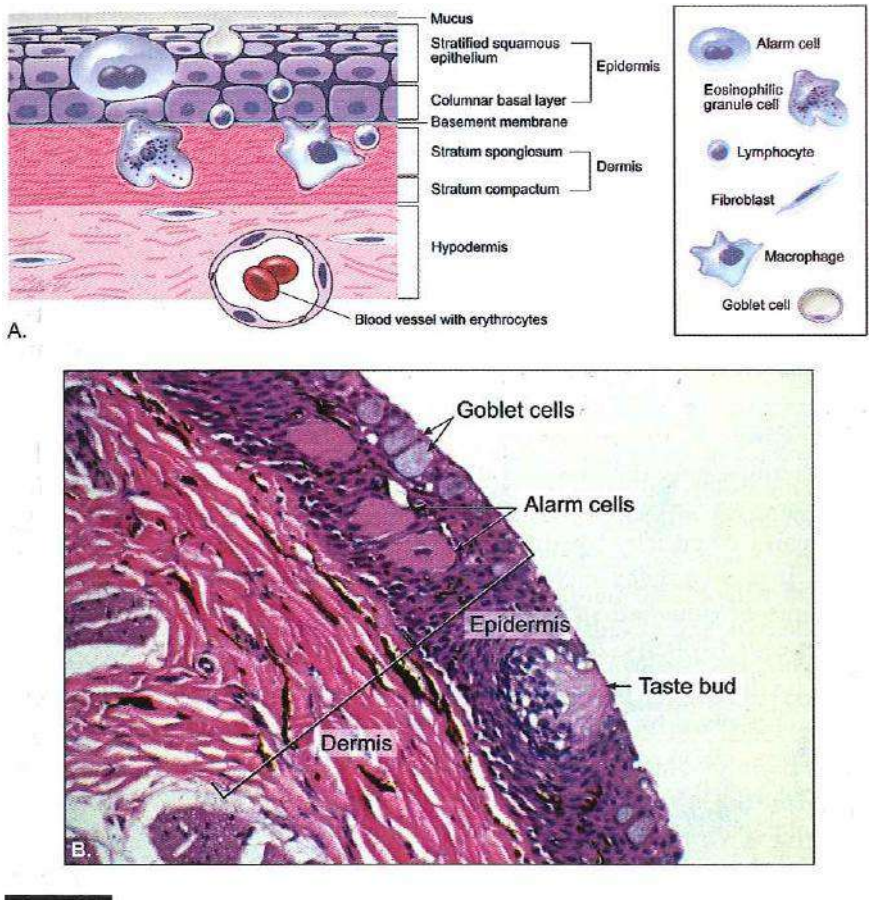
the mucosa, which consists of epithelium, a lamina propria of fibrous connective tissue, and sometimes a muscular mucosa. Between the mucous membrane and the muscularis, which consists exclusively of muscles, lies the submucosa, which consists of fibrous connective tissue. The outer layer of the serosa consists of fibrous connective tissue covered by a simple scaly mesothelium (Grizzle and Rogers, 1976).

The intestinal mucosa is considered to be an immunologically important site in bony fish (Cain *et al.,* 2000). In carp, the posterior segment of the gut, termed the second gut segment, plays an important role in mucosal immunity (Rombout and van den Berg 1989; Rombout et *al.,* 1989; Rombout *et al.,* 1989) and comprises 20-25% of the gut length (Rombout et *al.,* 1993; Press and Evensen, 1999). The gut-associated lymphoid tissue of most teleosts, including rainbow trout (McMillan and Secombes, 1997), carp (Rombout *et al.,* 1993), and perch (Picchietti et *al.,* 1997), consists of cells with lymphoid morphology located between the gut epithelial cells. They are mainly intraepithelial T lymphocytes (Bernard et *al.,* 2006; Huttenhuis *et al.,* 2006), but we also find lymphocytes mainly located in the lamina propria (Rombout *et al.,* 1993; Danilova and Steiner, 2002 ; Huttenhuis *et* al., 2006). Lymphoid aggregates resembling ileal or Peyer's patches in mammals are absent. The GALT of teleosts consists mainly of lymphocytes of various sizes, plasma cells, macrophages, and various types of granulocytes (Du Pasquier and Litman, 2000). Periodic acid Schiff (PAS) positive cells and eosinophilic granule cells are present and may serve to modulate immune hypersensitivity reactions occurring in the gut. In the intestinal epithelium and lamina propria, macrophages act as scavengers and antigen presenters. In carp, intestinal macrophages differ from macrophages isolated from other lymphoid organs in that they adhere poorly to glass and plastic, form clumps with lymphocytes, express antigenic determinants on their outer membranes, and bind immunoglobulins (Ig) (Rombout et *al.,* 1986, 1989a, b, 1993).

The hepatic biliary system begins with intracellular biliary tubules, which anastamose extracellularly to form bile ducts. These fuse in the gallbladder, which directs bile through the common bile duct to the intestine. The gallbladder is lined with transitional epithelium. Hematopoietic tissue with melanomacrophage centers is associated with larger hepatic blood vessels (Robert, 2001).

The skin

Fish skin provides protection from physical, chemical and biological damage. It consists of two anatomical layers, the epidermis and



A. Diagram of the basic anatomical structures of the skin and the identification and location of associated cells related to the immune system.

B. Photomicrograph of channel catfish skin (sensory barbel) (H&E stain).

dermis. The thickness of the stratified epithelium of the epidermis varies with body area, age, sex, maturation, and environmental conditions (Grizzle and Rogers, 1976; Yasutake and Wales, 1983). On average it is 10-12 cells thick. Cells in the columnar basal layer of the epidermis, called the stratum germinativum, replicate and migrate to the surface of the fish. This basement layer lies directly above a basement membrane. At least six cell types have been described in the epidermis of bony fish, including filamentous squamous cells (keratinocytes), mucus cells, chemosensory cells, club cells (alarm cells), granule cells, and chloride cells (Grizzle and Rogers, 1976; Yasutake and Wales, 1983; for an overview, see zaccone et *al.* 2001). Squamous cells are the most common in the epithelium.

These cells are rounded in shape with fiber bundles and mitochondria surrounding a generally ovoid nucleus (Roberts, 2001). At the epithelial surface, the keratinocytes flatten and their cytoplasm consists mainly of elongated vesicles, degenerated mitochondria, and denser fiber bundles. The outermost cell layer is not keratinized. The outermost cell surfaces have intricate microridges of unknown function that may help retain mucus secretions on the skin. Mucous cells begin to differentiate in the germ layer and migrate to the surface of the skin, where they release their contents. Mucus packets are bound by membranes and gradually fill the cell as they migrate to the surface. On the surface of the epithelium, the mucous cell (holocrine gland) moves between the keratinocytes and empties its contents. The epidermis is covered by a glycocalyx or cuticle composed of a thin layer of mucopolysaccharide (1.0 g). It is a complex mixture of molecules derived primarily from the contents of desquamated surface epithelial cells and the mucus secreted by goblet cells (Roberts, 2001).

The deep layers of the epidermis contain alarm cells and melanophores that do not reach the surface. The cell contents of the alarm substance are released in an oily form when the epidermis is physically damaged (Grizzle and Rogers, 1976). Capillaries extend from the dermal papillae into the epidermis and are located within 10 cell layers of the surface (Lobb, 1987).

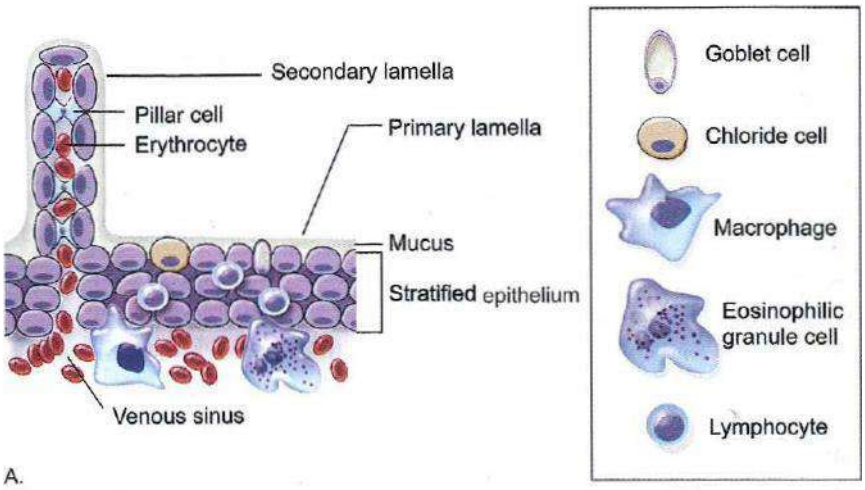
The dermis consists of two layers. The upper layer, called the spongy stratum, consists of a loose network of collagen and reticular fibers and is adjacent to the epidermal basement membrane directly above. It contains chromatophores, mast cells and scale bed cells. The bottom layer, the stratum compactum, is a dense collagen matrix that provides structural strength to the skin. The hypodermis, which is below the dermis, is made up of loose connective tissue. It is more vascular than the overlying dermis. Melanophores are present in the hypodermis, dermis, and sometimes in the epidermis.

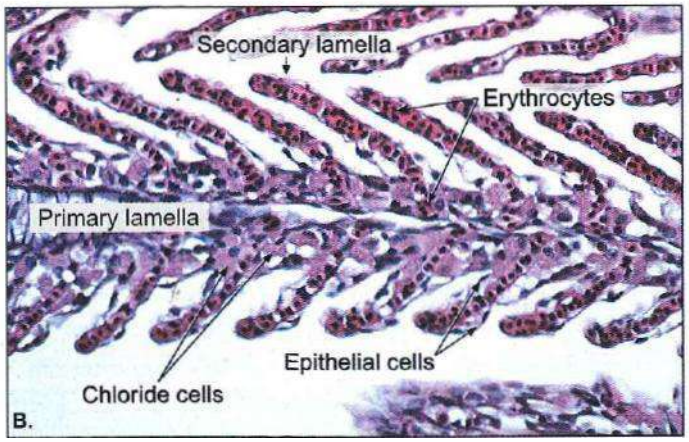
No organized lymphoid germinal centers were found in the skin (Flajnik, 1998), although cells with lymphocyte morphology could be detected by light microscopy in stained sections of catfish skin tissue (Lobb, 1987). These cells are present throughout the epidermis and are mainly located near the germinative layer at the junction of the epidermis and dermis (Lobb, 1987). Cells secreting specific and total antibodies (ASC) were isolated from catfish skin and detected with ELISPOT (Zhao et al., 2007). B cells isolated from channel catfish skin can be *stimulated with LPS to replicate and* secrete antibodies in vitro, a response which in turn is abolished by the addition of hydroxyurea to the culture medium (Zhao et *al.,* 2007). Macrophages are also present in the skin (Roberts, 2001).

Gills (Fig. 1.3)

The gills consist of gill arches, filaments (primary lamellae) and gill lamellae (secondary lamellae). Two rows of filaments are present on each arch, and the secondary lamellae branch off perpendicularly from the filaments (Grizzle and Rogers, 1976; Yasutake and Wales, 1983). The branchial arches and filaments are supported by a branching system of cartilaginous rods. A stratified squamous epithelium covers both the gill filaments and gill lamellae. The lamellae form the actual breathing surfaces. Each slat comprises a network of interconnected spaces separated and supported by columnar cells. Blood enters the lamellae from the afferent arterioles of the filaments and exits in the efferent arterioles. The lamellar intercellular spaces through which blood circulates are lined with endothelial cells. A basement membrane covers the endothelial cells and pillar cells that form supporting "protrusions" around the intralamellar spaces (Grizzle and Rogers, 1976). The stratified epithelium itself is only one to two cells thick to allow for gas exchange, a degree of thinness that leaves the tissue vulnerable to pathogen invasion.

Different cell types are associated with the gill epithelium. Chloride cells function in the transport of Cl and other ions across the epithelium. These cells are more spherical than the surrounding epithelial cells; they protrude slightly above the surface (Yasutake and Wales, 1983) and their cytoplasm is more eosinophilic (in hematoxylin and eosin stained sections) than in the case of other epithelial cells. Chloride cells are abundant in the epithelium of the gill filaments between the lamellae (Grizzle and Rogers, 1976). Mucus cells are abundant in the lamellar epithelium and appear under the light microscope as mucus-filled domes or vacuolated cells (Yasutake and Wales, 1983). Goblet cells are more commonly present at the edges near the arterioles. Alarm substance cells are absent from gill epithelia (Grizzle and Rogers, 1976). Although the surface of the branchial lamellar epithelium is irregular, it lacks the feature





A. Diagram of the basic anatomical structures of the gill epithelium and the identification and location of associated cells related to the immune system.

Photomicrograph of fish gills. Note capillaries with erythrocytes in secondary lamellae and chloride cells concentrated in lamellae pits (H&E stain).

Microcrests on the surface of the skin epidermis (Roberts, 2001). Nonetheless, these irregularities are sufficient to facilitate the fixation of mucus, which, in addition to its role in reducing microbial invasion, also serves to regulate the transfusion of gases, ions, and water across the epithelial membrane (Roberts, 2001).

Similar to the skin, there is no evidence for the existence of organized accumulations of lymphatic tissue in the gills. Nevertheless, a number of studies have demonstrated functional immunological activity of gills and gill- associated leukocytes and lymphocytes (Goldes et *al.*, 1986; Powell et al., 1990; Lumsden *et al.*, 1995; Davidson et al. al., 1997 ; Lin *et al.*, 1998; Rombout *et* al., 1998; Dos Santos *et* al., 2001 a, b). Considerable numbers of lymphocytes, ASCs and macrophages have been found in the gill tissue of Atlantic salmon and flounder (Lin et *al.*

1998). In leukocyte suspensions from carp gills (as in the skin), Rombout et al. (1998) found a rich population of intraepithelial lymphocytes (IEL) that reacted with a monoclonal antibody (mAb WCL38) specific for IEL T cells in carp gut. In IEL gill leukocyte suspensions, WCL38 + cells made up the bulk of the lymphoid cell population. Lymphocytes with surface immunoglobulin (ie, B cells) were a minor component of these cell populations. Numerous WCL38 + cells were detected at the base of the gill lamellae in cryosections. Immunogold staining showed that WCL38 + cells had the ultrastructure of lymphoid cells, although two morphologically distinct cell types were found: small lymphocytes with a high nucleus-to-cytoplasm ratio and larger granular lymphocytes with a nucleus-to-cytoplasm ratio and a variable amount of electrons. -dense, lysosome-like material.

3.2 ONTOGENY OF MUSCLE LYMPHOCYTES AND RELATED IMMUNE CELLS

Lymphocytes and other cells (such as macrophages) involved in acquired teleost immune responses are present in gut-associated immune tissues and other mucosal tissues and most likely evolved at these sites during the early development of the vertebrate adaptive immune response (Matsunaga, 1998 ; Matsunaga and Rahman, 1998; Cheroutre, 2004). However, in current teleosts, the ontogeny of mucosal lymphocytes has not been elucidated, and the extent to which they develop and reside in mucosal tissues or migrate to and from primary and secondary lymphoid organs such as the head, kidneys, and spleen remains to be determined. to be determined.

The mammalian gut can function as a primary lymphoid organ and intraepithelial lymphocytes (IEL) develop at this site (Lefrancois and Puddington, 1995) and, as mentioned above, it is likely that the early adaptive immune system of vertebrates also evolved in the gut epithelium (and possibly skin and gill epithelia) (Matsunaga and Rahman, 1998; Cheroutre, 2004). With the evolution of the adaptive immune system

However, the thymus acquired the mechanisms of lymphocyte maturation and selection and subsumed this function from mucosal sites. Thus, the intestinal mucosal tissues were ultimately relieved by the thymus of the responsibility of educating the developing ML in terms of self and non-self (Cheroutre, 2004).

The immune mechanisms of mucosal surfaces have been extensively studied in higher vertebrates, and the role of specific T and B cells located in the epithelia is being elucidated (Cheroutre, 2004). For example, in mice, epithelially localized pT cells have been shown to

migrate from GALT and peripheral lymphoid tissues following antigenic stimulation (Kim *et al.*, 1998). In this process, specialized cells in the follicular epithelium of the gut, called M cells, sample the lumen of the gut and transport antigens to the subepithelial tissues and the GALT (Neutra *et al.*, 1996). Local dendritic cells then process these antigens and then distribute them to peripheral lymphoid tissues, where resident naïve al3 T cells are activated and proliferate. These antigen-specifically differentiated T cells then migrate to the intestine, where they colonize the epithelium as effector and memory cells.

There are also specialized IELs in the mammalian gut that develop via an extrathymic pathway (Lefrancois and Puddington, 1995). These IELs consist mainly of)6 T cells with an oligoclonal TCR repertoire (Regnault *et al.*, 1994; Cherotre, 2004; Bernard *et al.*, 2006). The mechanisms responsible for the limited repertoire are unknown, but are thought to be the result of selection during lymphocyte development in the gut (Takimoto et al., 1992), a process involving the resident microflora (Helgeland et *al.*, 2004). It has been suggested that the development of extrathymic T cells also occurs in teleosts, at least in carp, a species in which the first studies on the ontogeny of mucosal lymphocytes were systematically performed (Huttenhuis *et al.*, 2006). These studies showed that IELs develop in embryonic intestinal epithelia prior to thymic development. In addition, it was found that Rag-1 expression in intestinal tissues occurs concomitantly with the early onset of these intestinal IELs. However, there may be species-specific differences between teleosts in terms of IEL ontogeny. A recent immunoscope-based analysis (Fannetier *et al.*, 1995) of IEL and systemic T cell receptor (TCR) VI3Jil spectra in trout showed that intraepithelial T cells isolated from the gut of naive fish express TCR Having repertoires similar to T cells found in blood and spleen (Bernard et *al.*, 2006). Although this finding does not rule out an extrathymic developmental pathway for IEL or a subpopulation of TELs not examined in this study, it does suggest (at least in trout) that other IELs correspond to random samples of systemic ar•T lymphocytes (Bernard et *al.*, 2006).

The predominant population of IEL in the mammalian gut consists of y8 T cells, which are thought to have evolved before af3 T cells in the development of adaptive immunity. Although genes encoding the y8 TCR have been identified in Japanese flounder (Paralichthys *olivaceus)* (Nam et *al.*, 2003), the extent to which teleost lymphocytes, which correspond to y8 T cells, are expressed in IEL Populations do not exist, still not known. Answering this question requires the development of reagents such as monoclonal antibodies to identify characteristic cell surface receptors and helper proteins in teleosts (Miller *et al.*, 1998).

T cells are present in mucosal tissues, but current evidence suggests that they develop in the primary lymphoid tissues of the primary kidney. In zebrafish, B cells appear first in the embryonic pancreas and then in the primary kidney (Danilova and Steiner, 2002). In carp, B cells first appear in the head, kidney and spleen of the embryos, then in the mucosal organs and thymus, but Ig $^{+}$ lymphocytes are never abundant in the thymus and intestine (Huttenhuis *et al.*, 2006).

3.3 INHERENT MUCOSAL IMMUNITY

The mucosal surfaces of the skin, gills, and intestines are constantly exposed to environmental pathogens; However, under normal circumstances, they remain free of infection and life-threatening lesions. Epithelia also heal quickly after mechanical or chemical injury. Resistance to infection and recovery from traumatic injury are facilitated by nonspecific innate immunity, which consists of a plethora of constitutively expressed elements as well as induced components of the inflammatory response. The physical factors of innate immunity consist of the membrane-anchored surface mucus barrier (glycocalyx) and the underlying contiguous epithelial cells with their tight junctions. The components of innate immunity can be broadly classified as cellular or humoral effectors.

3.3.1 Cellular components of the innate immunity of the mucosa

Teleosts have interacting leukocyte subpopulations that mediate both innate and adaptive immune responses (Miller et *al.*, 1998). Cell populations involved in the innate immune response include phagocytes Cells (macrophages and neutrophils), non-phagocytic cells (natural killer cells (NK) and non-specific cytotoxic cells (NCC)) and other cells (mast cells /eosinophilic granule cells and rod cells). Eosinophilic mast cells/ granule cells have similar structural and functional properties to mammalian mast cells (Reite, 1997) and store a range of inflammatory and antimicrobial compounds including phospholipids, alkaline phosphatase, peroxidase and lysozyme (Silphaduang et *al.*, 2006). Rod cells are present in the blood and epithelium of a large number of TV species (Reite, 1997, 2005) and have a characteristic morphology with club-shaped cytoplasmic crystal inclusions shed at the level of the epithelial, mesothelial, and endothelial surfaces. Although there are still questions about the origin and function of these cells, the most recent studies interpret rod cells as elements of the host's defense system that arise in connection with exposure to various stressors such as

parasites, neoplasia, viral infections and general tissues. (Reite, 1997, 2005; Manera and Dezfuli, 2004; Bielek, 2005; Reite and Evensen, 2006; Silphaduang et *al.*, 2006).

The innate cellular inflammatory response of teleosts is typically biphasic, beginning with an influx of neutrophils, followed by an influx of monocytes and macrophages (Sharp et al., 1991; Neuman *et* al., 2001). Neutrophils are derived from the main kidney, while macrophages are derived from blood-derived monocytes, which migrate to the affected tissue after an inflammatory attack. Monocytes develop from hematopoietic stem cells of the primary kidney and/or spleen. In addition to phagocytes, which extravaginate and migrate into tissues during inflammation, mucosal tissues also have resident macrophages involved in antigen uptake and antigen presentation, which are thought to play an important role in innate and adaptive immune responses (Huttenhuis et *al.,* 2006).

Gastrointestinal tract: The gastrointestinal tract of bony fish contains intraepithelial macrophages as well as neutrophils and mast cells/ eosinophilic granule cells (MC/ECG) located in the lamina propria (Georgopoulou and Vernier, 1986; Vallejo *et al.,* 1989; Rombout et al., 1989, 1993b; Davidson et al., 1991; Powell *et al.,* 1991; Dorin et *al.,* 1993; Sveinbjornsson et *al.,* 1996; Hebert et al., 2002; Leknes, 2002; Grove et al., 2006). In experiments performed on platy (Xiphophorus maculatus), ferritin from horse spleen injected into the coelomic cavity was taken up by macrophages mainly located in the lamina propria of the gut (Leknes, 2002). A submucosal MC/ECG layer is well developed in salmonids.

MCs/ECGs can migrate from the submucosal layers of the gut to the villi or mucous membranes, as marked degranulation of these cells also occurs in some allergic and bacterial infections. Experimental intracoelomic injection of extracellular products from culture supernatants of the bacterium *Aeromonas Salmonicida* caused vasodilation of blood vessels in the lamina propria of the intestine with concomitant proliferation and degranulation of MC/ECG cells (Ellis *et al.,* nineteen eighty-one). Rod cells are often associated with the presence of adult parasitic trematodes or cestodes in the gut and cystic helminth larvae in the gut or its adjacent tissues (Reite, 1997; Dezfuli *et al.,* 1998; Bielek, 2005).

A significant number of neutrophils (>64% of leukocyte cells isolated from collagenase-digested gut) appear to reside in the gut of healthy juvenile channel catfish, suggesting that innate immunity plays an important role in host defense in this species (Hebert *et al* 2002). Similarly, in sea bream *(Sparus* aurata), acidophilic granulocytes (considered equivalent to

neutrophils in this species) are mainly distributed in the lamina propria of the hindgut mucosa (Miilero *et al.,* 2007). These cells are thought to play an important role in innate immunity and immune surveillance, and studies have shown that giving probiotics to sea bream causes an increase in the number of these cells in the gut (Picchietti *et al.,* 2007).

Ig lymph cells are diffusely distributed in the intestinal epithelium. Although this population consists mainly of intraepithelial lymphocytes (IEL, mainly putative T lymphocytes), it is believed that NK cells are also present (Rombout *et al.,* 1993). Isolation of cytotoxic IELs from rainbow trout gut was isolated and functionally characterized for non-specific target cell killing. These cells did not contain cytotoxic granules resembling those observed in mammalian NK cells, suggesting an alternative mechanism for cell destruction (McMillan and Secombes, 1997).

The skin:

Macrophages, neutrophils and other granulocytes such as MC/ECG occur in the deeper layers of the epidermis, particularly in response to inflammatory events such as parasitic infections (Cross and Matthews, 1993; Buchmann, 1999; Reite and Evensen, 2006). In rainbow trout and channel catfish, migratory macrophages and lymphocytes are present in the skin (Lobb, 1987; Peleteiro and Richards, 1990). Activation of fish leukocytes in *vitro* causes the production of leukotriene B4, which in turn induces neutrophil migration (Hunt and Rowley, 1986). Macrophages and teleost neutrophils secrete interleukin 1, which affects other macrophages (Secombes and Fletcher, 1992). These signaling molecules likely play a role in the induction and activation of the cellular innate immune response in the skin.

Langerhans cells are antigen-capturing dendritic cells found in human skin with the ability to process antigen and present to lymphocytes (Koch et al., 2006). These cells have a typical granular cytoplasm and defined cell surface determinants. Reports of resident phagocytes scavenging antigens in bony fish skin are rare (Peleteiro and Richards, 1990), with a single reference to epidermal cells with membrane folds resembling Birbeck granules typical of human Langerhans cells (Mittal et al., 1980).. Although phagocytes with typical Langerhans cell morphology are apparently not present in the epidermis of fish, this does not exclude the possibility that dermal macrophages migrating through the basement membrane into the epithelium take up and process the antigen. In fact, phagocytic cells that share cell surface determinants with Langerhans cells (referred to as indeterminate or agranular dendritic cells)

exist in the human epithelium (Rowden et al., 1979) and are postulated to be dermal macrophage files derived from Monocytes originate, which migrate in the epidermis and develop into Langerhans cells" (expression of surface determinants and formation of Birbeck granules) under the influence of chemokine gradients and a specific epithelial microenvironment (Koch et al., 2006). It has been hypothesized that the migration of macrophages in the epidermis of fish may be the equivalent of these undifferentiated Langerhans progenitor cells seen in human skin (Peleteiro and Richards, 1990).

NK or NCC cells have not been reported in the skin, but it is possible that activated cells recruited into the peripheral blood from the main kidney end up at this peripheral site (Graves et *al.*, 1985).

Gills: In addition to the previously described epithelial cells, mucus-secreting goblet cells, and chloride cells, several types of leukocytes have been isolated from the gills of teleosts. Macrophages, eosinophilic granule cells (EGC) and neutrophils have been isolated and characterized in perfused gill tissue from Atlantic salmon and flounder (Liman:J/1 limanda) (Lin et al., 1998). In experiments on platy *(Xiphophorus maculates), intracoelom* injected horse spleen ferritin was taken up by macrophages located in the gill filament but not by the gill lamellae (Leknes, 2002).

Thus, while the main function of gill eater cells is thought to be to scavenge foreign matter and kill waterborne infectious agents, these cells also appear to be involved in the removal of foreign matter from the blood (Leknes, 2002). Although resident dendritic cells in the gill epithelium have not been described, gill macrophages most likely process and present antigenic material to lymphocytes to initiate a specific adaptive immune response (Davidson et *al.*, 1997; Lin et al., 1998).

3.3.2 Humoral components of innate mucosal immunity

The mucus layer of the skin, gills, and intestinal epithelium of fish is a complex mixture of molecules secreted by goblet cells and cellular contents released by crumbling surface epithelial cells. The main component of mucus is mucin, which consists mainly of glycoproteins. Also present are lysozyme, proteolytic enzymes and C-reactive proteins (Ingram, 1980; Fletcher, 1981). Slime acts as both a physical and chemical barrier against microbial invasion and environmental stress.

Non-immunoglobulin humoral defense factors in fish have been classified into four general categories based on their effects on invading pathogens: (1) inhibitors of microbial growth, (2) enzyme inhibitors, (3) lyricants (lysins), and (4) agglutinins/precipitins (Alexander and Ingram, 1992). Various antimicrobial compounds belonging to these categories, including trypsin, lysozyme, lectins, complement, and other lytic factors, are present in mucus and mucosal tissues where they serve to prevent adhesion and colonization of micropathogenic organisms (Alexander and Ingrain, 1992 ; Dalmo *et* al., 1997). These factors are described below with specific references to their role in mucosal innate immunity, where known.

Substances that inhibit microbial growth: Inhibitors of microbial growth - transferrin, ceruloplasmin, metallothionein and interferon - are all present in fish tissue (Alexander and Ingram, 1992). Transferrin is an acute phase protein that is triggered during inflammation to remove iron from damaged tissue and activate macrophages (Magnadottir, 2006). It is constitutively expressed in liver cells. Lactoferrin, a transferrin-related protein, is present in mammalian mucus secretions but has not been described in fish mucus or epithelial cells (Alexander and Ingram, 1992). Interferons (IFN) are secreted proteins that put cells into an antiviral state and induce the expression of Mx and other antiviral proteins (Leong et al. "1998; Robertson, 2006). Type I α- and 13 -IFNs and type II γ-IFNs have been identified or inferred in a number of different fish species (Graham and Secombes, 1990; Alexander and Ingram, 1992; Robertson, 2006). IFNγ produced in NK cells modulates innate immune responses ; but as mentioned, there are no studies showing whether or not NK cells are found in mucosal tissues.

Enzyme Inhibitors: The basic function of enzyme inhibitors is to maintain homeostasis of blood and other body fluids by regulating enzyme activities, including those involved in complement activation and coagulation functions (Alexander and Ingram, 1992). After pathogen invasion, destructive enzymes are actively secreted into tissues by the parasites and passively released from damaged host cells, including neutrophils and macrophages, that have migrated to the site of infection. These released proteases must be inactivated to prevent and reduce secondary tissue destruction. A variety of proteinase inhibitors (serine, cysteine, and metalloproteinases) have been isolated and characterized in mammals, but few have been described in fish. The most widely studied macroglobulin a2 in fish has broad inhibitory effects through encapsulation of protease molecules (Armstrong and Quigley, 1999; Magnadottir, 2006). The extent to which enzyme inhibitors act on the mucosal surface is currently unknown.

Lytic Agents: The lytic components of humoral innate immunity are enzymes that exist as single molecular entities, such as lysozyme, or as a cascade of enzyme components, such as those found in the complement system.

Lysozyme has been found in the tissues and secretions of fish, including the gut, dermal mucus and gills (Alexander and Ingram, 1992; Magnadottir, 2006), where it is produced by macrophages, neutrophils and eosinophilic granule cells (Murray and Fletcher, 1976). Lysozyme attacks structures containing N-acetylmuramamine and N-acetylglucosamine linked to 13 1-4 (the peptidoglycan constituents of bacterial cell walls) and the chiton, a constituent of fungal cells, and is therefore both antibacterial and antifungal. It also functions as an opsonin with subsequent activation of complement and phagocytes (Magnadottir, 2006). The amount of enzyme varies with tissue and fish species (Alexander and Ingram, 1992). Lysozyme has been described in the mucus of a number of fish species including carp and channel catfish.

The teleost complement system comprises more than 35 soluble plasma proteins that play a role in innate and adaptive immunity (Boshra *et al.,* 2006). Complement activation products initiate or are involved in the innate immune functions of phagocytosis and cytolysis of pathogens, solubilization of immune complexes, and inflammation (Boshra et al., 2006). There are few experimental studies addressing the extent to which components and functions of complement are present in mucosal tissues and secretions. A study showing that the parasitic monogenic trematode *Gyrodactylus salaris was killed* after incubation in Atlantic salmon skin mucus suggests that components of the complement system are involved in the innate immune response in the skin. In this study, mucus activity was about one -twentieth that found in serum. Activity (in serum) was not dependent on the immune status of the fish and opsonization of parasites with antibodies did not enhance killing, suggesting that complement may be mediated via the alternative pathway (Harris *et al.,* 1998) or via the lectin pathway (Buchmann, 1998, 1999). The transcripts of complement factors C3 (rainbow trout) and C7, P (FP), Bf/C2A, C4 and D (FD) (carp) have been detected in the skin after infection by the protozoan parasite *ciliate Ichthyophthirius multifliis* (Sigh *et al.,* 2004; González *et al.,* 2007 a, b). These studies also suggest that parasitic infection causes expression of a subset of extrahepatic complement genes in the skin. It is postulated that proteins are produced in macrophages (Buchmann, 1999).

Japanese eel *(Anguilla japonica) mucus* contains a locally produced hemolysin that may play a nonspecific protective role, although this has not been determined (Alexander and Ingram, 1992). Trypsin has been found in mucus and mucus-secreting cell layers of the skin, gill lamellae, and foregut of Atlantic salmon and rainbow trout, where it is thought to play a role in nonspecific immunity to microbial invasion on these surfaces (Hjelmeland *et al.,* 1983; Brown *et al.,* 1990). It should be noted that the presence of active trypsin on these surfaces indicates that enzyme inhibitors are not present.

Agglutinins: Agglutinins are agglutinating factors (not immunoglobulins) that are produced in the absence of defined antigenic stimuli (Ingram, 1980). These carbohydrate-binding proteins cause opsonization, phagocytosis, and activation of the complement system (Buchmann, 1999). Slimy agglutinins and precipitins consist mainly of lectins, such as C-type lectins and pentraxins. In the presence of Ca^{+}, mannose, N-acetylglucosamine, and fucose bind, resulting in opsonization, phagocytosis, and activation of the complement system (Magnadottir, 2006). Pentraxins, which comprise C-reactive proteins, are commonly implicated in the inflammatory response in the acute phase and participate in innate immunity by activating complement pathways. A hemagglutinin is present in the mucus of Japanese eels, but the extent to which it is involved in innate immunity is not known (Magnadottir, 2006). Lectins present in skin mucus appear to play a role in the innate immune response against skin parasites such as the ciliates L. *multifiliis* and the trematode Gyrodactylus (Yano, 1996; Buchmann, 1999; Buchmann et al., 2001; Xu et al., 2001). However, in many cases the roles of mucus lectins remain unsolved and it is possible that they function independently or in cooperation with other biologically active molecules (Alexander and Ingram, 1992).

Natural Antibodies: Although antibodies (immunoglobulins) are generally considered to be the primary effector mechanism of the humorally acquired immune response, natural antibodies are also considered components of the innate immune system. There are several sources of natural antibodies including: adoptive transfer, exposure to environmental antigens, and production by gene rearrangement without specific antigen stimulation (Sinyakov et al., 2002; Magnadottir, 2006). Natural antibodies have been increasingly shown to play a role in mammalian immunity, and their presence and role in fish immunity is also well documented (Sinyakov et *al.,* 2002; Magnadottir, 2006). The fact that specific antibodies are produced locally in mucosal tissues suggests that natural antibodies may also be present at these sites, although no systematic studies have been conducted to determine this. However, in channel

catfish vaccine studies, a relatively low but consistent number of antibody-secreting cells (plasma cells) expressing antibodies to the major surface antigen of I. *multifiliis* were detected in the skin epithelium of naïve fish (Dickerson, unpublished data). These could be natural antibodies. Given the importance of the surface mucosa as the first line of defense against pathogens, it seems logical to expect natural antibodies to be present at these sites. There is a need for further research in this area.

Antimicrobial peptides: Low molecular weight antibacterial peptides are typically associated with peripheral blood leukocytes or mucosal surfaces in vertebrates (Bevins, 1994; Cole *et al.*, 1997; Smith *et al.*, 2000; Silphaduang *et al.*, 2006). They exhibit a number of properties useful for innate immune responses, namely a broad spectrum of activity against microorganisms, low toxicity to host cells, ease of synthesis, and rapid rates of diffusion (Smith *et al.*, 2000). Antimicrobial peptides have been described in the skin of a number of different fish species, including rainbow trout, where mucilage extracts have demonstrated muramidase and non-muramidase lytic activity against selected bacteria (Smith et *al.*, 2000; Ellis, 2001).. The peptide piscidin has recently been found in a variety of bony fish species and is produced in gill, dermal, gastric and intestinal epithelia. Piscidin is produced in MC/eosinophils and rod cells (Cole et *al.*, 1997; Silphaduang *et al.*, 2006). The presence of piscidins in eosinophils present in epithelial tissues suggests that they play an important role in the innate defenses of these tissues (Silphaduang *et* al., 2006).

3.4 ADAPTIVE MUCOSAL IMMUNITY

The adaptive mucosal immune response of teleosts, thought to have arisen early in the development of acquired immunity, plays an important role in protecting against infection. Fish are the first vertebrates to possess both innate and adaptive immunity, and acquired immunity is postulated to have evolved earliest in the gut of jawed fish (Matsunaga, 1998; Matsunaga and Rahman, 1998, Cheroutre, 2004). However, relatively few immunologists have focused their efforts on studying mucosal immunity in fish, and therefore there is much less background knowledge compared to what is known about the mammalian system. Furthermore, experimental data from fish are in many cases necessarily descriptive rather than mechanistic due to the lack of immunological reagents for quantitative studies (e.g. antibodies to cell surface antigens and signaling molecules and isogenic knockout animals) (Rombout et *al.*, 1993; Lin *et al.*, 1998; Huttenhuis *et al.*, 2006). For example, although it is known that antigen is preferentially absorbed in the hindgut (Rombout *et al.* 1985; Georgopoulou and

Vernier 1986; Otake *et al.* 1995), the precise sites where antigen is processed and presented by phagocytes and where B and T -Cells interact, proliferate and differentiate remain unknown. Relatively few cell signaling molecules such as cytokines and chemokines have been identified. Antibody-secreting lymphocytes and plasma cells have been described in the intestinal epithelium and lamina propria (Rombout *et al.*, 1993; Hebert *et al.*, 2002), but the extent to which phagocytes and lymphocytes divide between the periphery (mucosa) and the central (pronephros and spleen) is largely undetermined.

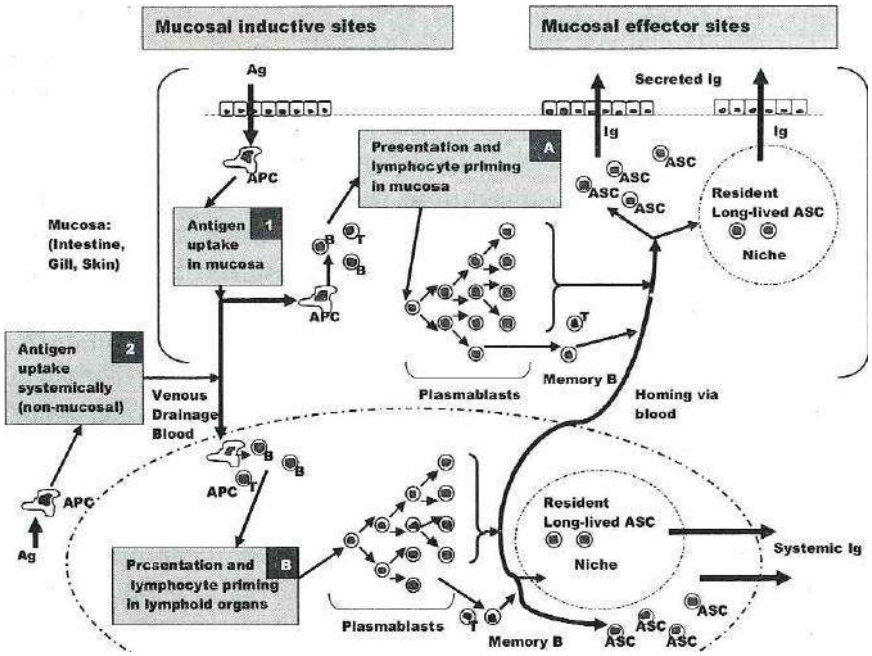
3.4.1 Induction and initiation of mucosal adaptive immunity (Fig. 1.4)

There is experimental evidence that induction of mucosal immunity occurs through mechanisms similar to those that exist in higher vertebrates, namely antigen processing and presentation by phagocytic cells, followed by priming of B cells and **T cells,** induction of B -cell proliferation and differentiation with the help of T-lymphocytes and the production of antibodies by fully differentiated plasma cells (Miller et *al.*, 1998). However, the exact sites of antigen, induction, and the extent to which the mucosal and systemic immune responses interact are still unknown. The following sections present the current knowledge and hypotheses regarding the induction of mucosal immunity in the different mucosal tissues of teleosts.

Gastrointestinal tract: The initiation of the mucosal immune response begins with the absorption of the antigen. The distal gut of teleosts (referred to as the second gut segment) is the primary site of antigen uptake, and enterocytes in this region are thought to function similarly to specialized membranous epithelial cells (M-cells) found in the gut, as fundamentally as the Ways in which antibodies are produced at mucosal sites and translocated through intact epithelial cell layers also remain unanswered. It is clear that far fewer data are available for fish compared to the substantial body of experimental data that has contributed to elucidating the basic mechanisms of mucosal immunity in mammals. Most experimental work on basal immunity in fish has focused on the systemic immune response, and what is known about mucosal immunity has been gleaned mostly from studies of the fish gut, with less information available on gills and skin.

Since the elements of bony mucosal immunity are outlined in each section below, the mucosal immune response in mammals will be briefly reviewed, where appropriate, to highlight the notable anatomical and functional differences (or similarities) that exist between the two groups. However, it should be emphasized that modern-day fish have an adaptive mucosal

immune system that is as effective as that of mammals at preventing infection. Comparative immunological studies aim to shed light on evolutionary adaptations and provide information on common and unique mechanisms that exist between these different groups of animals.



Coward. 1.4 Conceptualized elements of adaptive mucosal immunity in teleosts.

In this model, derived from various studies in different fish species, it is hypothesized that **mucosal (1) and systemic (2) exposure to** antigen **(Ag) elicits a** mucosal antibody **(Ab)** response. Mucosal contact with antigen can also trigger the production of systemic antibodies. After entering through the mucosal epithelium or systemically (eg, inoculation), antigen is phagocytosed by antigen presenting cells **(APCs),** processed, and presented in putative mucosal inducing sites **(A)** and/or the central inducing sites of the renal pulp, the anterior nephros and the spleen **(B).** Plasmablasts generated by T lymphocytes contribute to blood circulation from the renal pulp and spleen to peripheral mucosal sites. It is postulated that plasmablasts generated at mucosal induction sites can also circulate to central lymphoid organs. Upon contact with surface antigen, mucosal antibody responses can be induced without the production of systemic antibodies. Memory **B** cells, long-lived antibody **(ASC)-secreting cells,** humoral memory, and long-lived ASC niches are discussed in the text.

Mammals (Davina *et al.*, 1982; Egberts *et al.*, 1985; Rombout and van den Berg, 1989; Rombout et *al.*, 1989). M cells, which are modified intestinal epithelial cells, serve as sites of antigen uptake (Egberts *et al.*, 1985; McLean and Donaldson, 1990) and have apical membranes with

shorter and wider microvilli than those surrounding enterocytes (McLean and Donaldson, 1990). Epithelial cells of similar morphology have not been described in fish, but functional aspects of the posterior segment of fish gut suggest analogous roles for gut cells in this region, namely the ability to absorb proteins intact and the tight association of lymphoid cells (Rombout et *al.,* 1985).

Macrophages take up antigen from the posterior region of the gut, suggesting that this is a site of induction and initiation of the mucosal immune response (Rombout *et al.,* 1985; Doggett et al. Harris, 1991). Lymphocytes (termed intraepithelial lymphocytes or IEL) are diffusely distributed within the columnar epithelium (Rombout *et al.,* 1993; McMillan and Secombes, 1997; Picchietti *et* al., 1997.). They are mainly T cells expressing the posterior T cell receptor (TCR), but some antibody-secreting plasma cells are also present (Scapigliati *et al.,* 2000; Bernard et *al.,* 2006). Macrophages and lymphocytes are also diffusely distributed in the underlying lamina propria. Functionally and morphologically organized germinal centers comparable to ileum and Peyer's patches and regional lymph nodes of mammals are absent. Resident macrophages in the intestinal epithelium have been shown to take up antigen and present antigenic determinants on their outer membranes, suggesting an antigen-presenting function (Rombout and van den Berg, 1989). Differentiation and proliferation of resident or circulating antigen-specific 4th TB lymphocytes could occur locally after antigen preparation by resident macrophages, although this has not been demonstrated experimentally. The population of ap T cells found in IEL populations has been found to share functional and phenotypic similarity to 00 T cells found in the peripheral circulation (Bernard *et al.,* 2006), allowing for the possibility that **IEL** circulates in the blood. It is also possible that after the antigen has been taken up and processed (in the gut or elsewhere), the antigen-presenting cells migrate to the central lymphoid organs of the anterior nephros (also called the main kidney) and from the spleen, where they then present the antigen. to initiate the immune response (Rombout and Van den Berg, 1989). This latter possibility would predict that differentiated T cells, plasmablasts, or plasma cells, arising and developing in central lymphoid organs, circulate via the blood to peripheral epithelia. Again, there is no direct experimental evidence to determine where induction sites occur. However, studies indicate that anal administration of particulate bacterial antigen elicits both mucosal and serum antibody responses (Rombout et al., 1989).

Skin *and Gills:* The skin is the site where the immune system encounters most environmental pathogens (Kupper, 2000), and in mammals it has been postulated to serve as

an immune organ (Puri et *al.,* 2000). Mammalian skin contains phagocytic dendritic cells (Langerhans cells) that extend pseudopodial processes between epithelial cells to reach near the surface. These cells monitor the epidermal barrier to detect the intrusion of foreign antigens. Once an antigen is found, internalized, and processed, Langerhans cells migrate to regional lymph nodes for further development, which involves producing additional co-stimulatory molecules (involved in T cell activation) and stopping antigen processing (Kupper, 2000). The mature Langerhans cell no longer processes the antigen to ensure that only the antigen initially found in the skin is presented to trigger the immune response. The antigen is then presented to resident T cells, which, when activated, return to the skin to eliminate or prevent further antigen penetration (Kupper, 2000). In mice, the epidermis also contains a small number of specialized T cells &y called dendritic T cells. These cells have a restricted pattern of TCR utilization and appear to play a unique role in the skin's immune responses. Similar cells are not found in humans (Bogen, 2004).

Fish have phagocytes and leukocytes associated with the skin and gill epithelium, either within or just below the epithelium (Lobb, 1987; Iger and Wendelaar Bonga, 1994; Davidson *et al.,* 1997; Lin et al., 1998; Moore *et al.,* 1998), and these cells are thought to be involved in the initiation of the mucosal immune response. Cells with the morphology of mammalian dendritic cells have not been described in fish, but cells with analogous antigen presentation and processing are believed to exist based on evidence such as the relatively high expression levels of MHC II chain mRNA in the gills of Atlantic salmon (Koppang *et al.,* 1998). However, the exact sites of induction of mucosal immunity are unknown. Studies in sea bass have shown that dip vaccination elicits large numbers of antibody-secreting cells in the gills, with no concomitant response in the gut or systemic organs (Dos Santos et al., 2001b). Similarly, in channel catfish, vaccination by immersion in a soluble antigen has been shown to elicit a mucosal antibody response without stimulating a serum antibody response (Lobb, 1987). These studies suggest that the development of mucosal and systemic immune responses are separate at some level, although induction of an immune response has been postulated to occur at a specific mucosal level the site also causes stimulation in other distant mucosal tissues (Kawai et al., 1981, Rombout et *al.,* 1989; Davidson *et al.,* 1993). In fact, according to several studies on different fish species (St. Louis-Cormier et *al.,* 1984; Rombout *et al.,* 1989; Cain *et* al., 2000; Maki and Dickerson, 2003), there seems to be a definite communication between mucosal and systemic Induction sites after immunization at each site. For example, lymphocyte-containing antibodies were increased in rainbow trout skin after intracoelomic (ic) injection of sheep erythrocytes (St. Louis-

Cormier *et al.,* 1984). Similarly, ic injection of the surface antigen of the major parasite I. *multifiliis* in channel catfish induces both serum and skin antibodies (Maki and Dickerson, 2003). The migratory pathways of antigen presenting cells and lymphocytes in the epithelia and between these tissues and the anterior nephros and spleen are believed to be as described above for intestinal MALT. Research is needed to further elucidate the sites and kinetics of induction after antigen challenge at different sites. The various possible sites of antigen presentation are shown schematically in Figure 9.4.

3.4.2 Effector Mechanisms of Adaptive Mucosal Immunity

The effectors of adaptive immunity are antigen-specific antibodies and cytotoxic T cells, both found in teleosts. Although there is considerable experimental data on the molecular characterization of antibodies and the kinetics of antibody expression, much less information is available on antigen-specific cytotoxic T cell subsets (Nakanishi *et al.,* 2002a). Most of the experimental work on T lymphocytes has focused on lymphocytes isolated from peripheral blood, the head kidney (pronephros), or the spleen. Therefore, the information presented below on the effector mechanisms of mucosal adaptive immunity focuses on mucosal antibodies, B cells and antibody-secreting plasma cells.

Mucosal Antibodies: Mammalian mucosal antibodies, which are mainly dimeric molecules of the IgA isotype, are transported through the epithelial layers to the mucosal surface by the polyclonal Ig receptor (pIgR), which encodes the connecting chain (chain I) of IgA - and IgM molecules binds.. Part of the pIg, termed the “secretory component”, is released together with the Ig in the mucus secretions (Bogen, 2004).

In teleosts, the predominant antibody found in both mucus and blood is an IgM tetramer with a molecular mass in the range 600-900 kDa, with each monomeric subunit composed of two light chains (each polypeptide light chain - 25 kDa) and two heavy chains. Chains (any polypeptide heavy chain - size 70 kDa) (Wilson and Warr, 2002). Although they generally exist in tetrameric form under physiological conditions, fish Igs exhibit a degree of structural heterogeneity that results from uneven disulfide polymerization of monomeric or semimeric subunits (a light chain and a heavy chain) (Kaattari *et al.,* 1998 ; Bromage *et al.,* 2004). This diversity is not associated with isotypic differences (Bromage, 2005). Fish IgM is comparable to the pentameric mammalian IgM molecule in terms of heavy chain size, antigen affinity and avidity (Bromage, 2005).

J chains and pIg receptors have not been reported in teleosts, except in an early study in a marine fish, *Archosargus probatocephalus,* which described a 95 kDa molecule covalently linked to the heavy chain dimer Ig, the was isolated from skin mucus (Lobb and Clem, 1981). However, recent studies in pufferfish *(Takifugu* rubripes) (Hamuro *et al.,* 2007) and carp (Rombout *et al.,* 2008) suggest the expression of porcine receptors in the skin and other mucosal tissues of teleosts; and a function in the secretion of Ig.

Although tetrameric IgM is the most commonly produced antibody *in vivo* among different fish species and the only isotype shown to be an effector of protective immunity, this is new. Isotypes have recently been discovered. These include two transcripts [t. Genes encoding IgD antibodies in salmon, 8 genes encoding IgD antibodies in salmon, channel catfish, cod and Japanese plaice, and 0) and 'c encoding IgZ and IgT genes in zebrafish and trout, respectively (Bromage, 2005). Whether the functions of these isotypes occur in mucus secretions has not yet been elucidated.

Twenty years ago, a fundamental question unanswered in fish was whether mucosal antibodies are produced locally in the mucosa or distantly in the head, kidneys and spleen (Lobb, 1987). Today, experimental evidence suggests that they are produced locally (Lobb and Clem, 1981a,b; Lobb, 1987; Rombout *et al.,* 1993; Lin et *al.,* 1996; Cain *et al.,* 2000; Maki and Dickerson, 2003).. For example, a localized cutaneous antibody response is generated against I. *multifiliis,* a protozoan parasite that infects epithelial tissues in the skin and gills (Clark *et al.,* 1992). Passive immunization experiments in naïve catfish have shown that mouse monoclonal antibodies (mAbs) to i-antigens confer protection against a lethal parasite challenge (Lin *et al.,* 1996), but the antibodies must be present at the site of infection. The availability and function of antibodies depended on the molecular size of the antibody since mouse IgG but not IgM protected the antibodies. Also serum antibodies from actively immunized fish, which are tetrameric IgM-like molecules about 750,000 daltons (Wilson and Warr, 1992), failed to protect even after passive transfer in naïve animals, although such antibodies strongly immobilize the parasite *in vitro* (Lin et *al.,* 1996). Immobilization capacity *in vitro* corresponds to protection in *vivo* (Clark *et* al., 1995). These results indicate that antibodies must be present in the skin and presumably in the gills, where the parasite becomes infected, to provide protection.

In other studies using the I. *multifiliis infection system, a* two- to three-fold increase in IgM mRNA expression in the skin was detected at days 4 and 6 after I invasion. *multifiliis,* meaning upregulation of Ig transcription in response to infection (Sigh *et al.,* 2004): These results

suggest that antibodies are produced in the skin by resident antibody-secreting cells (ASC). Additional experiments directly demonstrated that antibodies against I. *multifiliis* are produced in the skin (Xu and Klesius, 2003). Skin explants taken from immune fish and placed in sterile tissue culture media produced antibodies specific for I. multifiliis that persisted for four days, suggesting that the skin cells were actively producing this specific antibody. Cultures of skin explants from immune fish but not control fish contained antibodies that immobilized I. *multifiliis* and reacted with the predominant surface antigen on Western blots. In addition, similar experiments showed that cutaneous antibodies against E. *columna4 are detected* in cultures of skin explants from infected channel catfish, suggesting that the antibodies are also involved in protective immunity against this bacterial pathogen (Shoemaker et al. al., 2005).

While experimental evidence suggests that mucosal antibodies are produced locally, the extent to which they differ in structure and function from serum antibodies remains unclear. Research has shown that antibodies from skin mucosa are physically and immunologically identical or share similar molecular epitopes with those isolated from blood (Lobb and Clem, 1981, 1982; St. Louii: Cormier *et al.,* 1984; Itami et al., 1988; Rombout et *al.,* 1993). However, carp studies using mAbs against purified Igs from mucus or serum revealed antigenic differences between skin mucosal and serum antibodies (Rombout *et* al., 1993). It has been suggested that alternative forms of Ig could be generated on mucosal surfaces that cannot be detected with current methods (Cain *et al.,* 2000; Bromage, 2006).

B Cells *in Mucosal Tissues:* In mammals, B cell differentiation is initiated by antigen presentation in secondary lymphoid tissues such as lymph nodes, mucosa-associated lymphoid tissue (MALT), and spleen. These lymphoid organs are organized to recruit naïve B and T lymphocytes from the blood and promote their interaction with the cognate antigen by migrating to these sites activated antigen-presenting cells from surrounding tissues. Once the lymphocytes have been activated and grown by cloning in centralized lymphoid organs, the resulting effector cells migrate and localize to infected or inflamed tissues. For example, B cells that are responsive to respiratory pathogens are first detected in local lymph nodes that drain the airways and then in the lungs (Moyron-Quiroz et *al.,* 2004).

Mucosal surfaces are particularly susceptible to infection because these epithelial surfaces are thin, permeable barriers within the body and the vast majority of infectious agents enter through these routes. In mammals, mucosa-associated lymphoid tissue is organized to respond to pathogens invading mucosal surfaces. In fish, the skin, gills and intestines represent the main

surfaces of the animal that are directly exposed to the environment and are therefore the entry point for many pathogens. It is possible that the lymphocytes and ASCs that lie just beneath these surfaces serve as the primary site for antigen presentation to B cells and are therefore a site for memory B cells to differentiate and proliferate, prompting a rapid response easier to reinfect.

Immune response in mammals, and a several orders of magnitude increase in IgG antibody affinity results from clonal selection of B cells (Gourley *et al.*, 2004). Only IgMs are produced in Teleosts and there is no class switching. Be it affinity maturation and somatic hypermutation (SHM). IgM in fish has been debated, but recent reports clearly indicate that a modest increase in antibody affinity for trout IgM and shark IgNAR occurs after immunization with model antigens (Cain *et al.*, 2002; Kaattari, 2002; Dooley, 2006). Sequence analysis of channel catfish heavy chain cDNAs revealed SHM of the $_{\text{VH and JH coding}}$ regions (Yang *et al.*, 2006). In mammals, cytidine deaminase (AID)-induced activation. is an essential mediator of somatic hypermutation, class switch recombination, and gene conversion, all of which occur during the process of B cell differentiation and affinity maturation. AID is exclusively expressed in germinal centers and appears to be the only specific B cell component required for these processes. AID has been shown to be expressed in the skin of channel catfish, suggesting that B cells can mature locally in the skin of this species (Saunders and Magor, 2004). Undifferentiated B cells responsive to LPS stimulation were isolated directly from channel catfish skin (Zhao et *al.*, 2008).

3.4.3 Immunological memory and mucosal immunity (Fig. 1.4)

Activated B cells differentiate into populations of memory B cells and antibody-secreting cells (ASCs), which include plasmablasts, short-lived plasma cells, and long-lived plasma cells. In mammals, long-lived plasma cells reside in the bone marrow, where they produce most of the circulating serum antibodies (Manz et al., 2002). Long-lived CSA can also occur in mucosal tissues (Etchart *et al.*, 2006). Long-lived plasma cells and memory B cells provide humoral immunological memory (Bernasconi *et al.*, 2002; Gourley et *al.*, 2004). Recent studies have provided evidence that antibody-secreting lymphocyte subpopulations similar to those in mammals also exist in fish (Bromage *et al.*, 2004). This work showed for the first time that long-lived ASCs reside in the head kidney of trout and that these cells accumulate in this tissue and secrete antibodies up to 35 weeks after immunization. These cells are a source of serum antibodies. These long-lived ASCs were not found in the spleen or in the peripheral blood (PBL)

population. Short -lived (ie, weeks) plasma cells were found in both the spleen and the large kidney.

As previously mentioned, there are no tissues in fish comparable to mammalian lymph nodes, and apart from the spleen and anterior nephros, the anatomical sites at which B cells encounter foreign antigen are not well defined (Bromage et *al.,* 2004). Recently, fish B cells have also been shown to have potent phagocytic and microbicidal activities not observed in mammalian B cells (Jun et al., 2006), suggesting that they may play an even more central role in initiation of immune responses than before. hypothesized., These findings raise questions as to where primary adaptive immune responses occur after infection and where memory B cells and long-lived plasma cells are generated and ultimately reside. It is possible, although untested, that the skin, gills, and intestinal epithelia with their associated lymphoid tissues are major sites of B-cell antigen presentation for epithelial pathogens. However, they may not be the exclusive sites, since infection of the skin with I. *multifiliis* (for example) results in the production of antibodies in both the skin and serum, showing that ASCs are present on both the skin and the kidney of the head are localized after infection (Maki and Dickerson, 2003). Nonetheless, it is possible that the tissues just beneath the epithelial surfaces serve as sites of antigen presentation for B cells and are therefore reservoirs for memory B and T cells, facilitating a rapid response to reinfection, although as noted above this is so, the case continues to be tested.

Whether long-term mucosal humoral immunity is provided by long-lived plasma cells remains an open question in mammals (Etchart *et al.,* 2006; Heipe and Radbruch, 2006). ASCs, located in the nasal mucosa, contribute to both serum antibodies and secretory mucosal IgA. Their longevity suggests that plasma cell survival niches exist in mucosal tissue and that these ASCs represent a second group of long-lived plasma cells (not localizing to bone marrow) that contribute to humoral immunity at the mucosal surface level. It is possible that a similar situation exists in fish. For example, channel catfish immunized against I. *multifihis remain* immune to surface infections for more than a year, suggesting that long-lived resident ASCs continuously produce protective cutaneous antibodies (Burkart *et al.,* 1990; Zhao *et al.,* 2008).

3.5 MUSCLE IMMUNITY AND VACCINES

A recent survey of the fish community shows that commercially available vaccines against 15 bacterial diseases are used in aquaculture worldwide (Hastein *et al.,* 2005). The two main methods of vaccination are immersion and injection. Oral vaccination is less efficient

compared to other methods, although an experimental method using a plant expression system has recently been developed that can increase the efficiency of this pathway (Companjen et *al.*, 2005). Immersion vaccination with inactivated bacteria or subunit antigens is used against the following bacterial diseases (Hastein *et al.*, 2005; Navot et *al.*, 20Q5): classical vibriosis *(Listonella anguillarum* or *Vibrio ordalii)* in bass, salmonids, catfish, ayu and turbot ; Furunculosis *(Aeromonas salmonicida)* in salmonids, bettas and goldfish; yersiniosis *(Yersinia ruckeri)* in salmonids, carp, eels, sole and sturgeons; Urellose paste *(Photobacterium damselae)* in sea bass and sea bream; warm water vibriosis *(Vibrio alginolyticus, V. parahaemolyticus, V. vulnificus)* in barramundi, grouper, sea bass, sea bream and snapper; Edwardsiella ictaluri *(Edwardsiella ictaluri)* in channel catfish; flavobacteriosis *(Flavobacterium columnare)* in salmonids; flexibacteriosis *(F. maritimus)* in salmonids and turbot; and Streptococcus *(Streptococcus iniae)* in rainbow trout, tilapia, turbot, and yellowtail flounder.

Viral vaccines approved for aquaculture are all based on inactivated antigens in oil emulsions. Because the viruses or subunit components do not replicate and are non-infectious, these vaccines are administered by injection (Biering *et* al., 2005). Antibodies are the main response elicited after immunization with these vaccines, which may not provide the most effective protection. Live attenuated virus vaccines include naturally occurring isolates with low virulence or viruses that have been otherwise attenuated. The advantage of these types of vaccines is that they can naturally infect and replicate in the host. Therefore, they can be administered either by immersion or orally. The main disadvantage is the risk of reversion by mutation to virulent forms (Biering *et al.,* 2005). However, no virus vaccine is currently administered to fish by immersion (Navot *et al.,* 2005).

Dip vaccination has been used effectively to protect fish from bacterial pathogens for many years, although the exact mechanisms of antigen uptake and protection in many cases are still unknown. However, in some cases it has been found experimentally that the antigen passes through the skin and gill epithelia directly or after hyperosmotic and/or ultrasonic treatment to reach the blood and lymphoid tissues (Alexander *et al.,* 1982, Ototake, 1996; Ototake *et al.,* 1996; Moore et al., 1998; Navot et *al.,* 2004). Ultrasound irradiation causes microscopic lesions of the skin (Navot *et al.,* 2004, 2005) and this treatment has been suggested to be comparable to intradermal immunization, which is one of the most effective vaccines in mammals (Navot *et al.,* 2005). Absorption is also improved after mild, controlled puncture or skin abrasion (Nakanishi *et* al., 2002b).

Dip vaccines against pathogens that enter through gills or skin epithelia have been effective when antibodies against surface antigens that block pathogen entry and colonization are induced. For example, dip vaccines against *Photobacterium damselae* subspecies *piscicida* (*formerly Pasteurella piscicida),* which consist of the overexpressed bacterial proteins 97-IcDa and 52-kDa, are effective with relative percent survival rates (RPS) of 50% compared to controls (Barnes *et al.*, 2005). Following immersion immunization of sea bass, the gills were found to be the primary sites of ASCs, suggesting that protective antibodies are produced (and possibly stimulated) locally (Dos Santos et al., 2001b; Barnes *et al. al.*, 2005). An experimental non-commercial subunit vaccine composed of the major surface antigen of *I. multifiliis elicits* a cutaneous antibody response and protective immunity to challenge (Wang and Dickerson, 2002; Wang et al., 2002). *and Methods of Administration that Enhance* Mucosal Immunity: Adjuvants are compounds that enhance immunity through accelerated, prolonged, or enhanced responses to vaccine antigens. Although many different adjuvants have been tested on fish (mainly by trial and error), water-in-oil immersions in mineral or non-mineral oils have been found to be the most effective in commercial aquaculture (Schijns and Tangeras, 2005). However, little information is available in the literature on adjuvants and mucosal immunity. Approaches used in the field of human vaccinology include the use of Toll-like receptor agonists (e.g. CpG motifs and glycans) as well as immunostimulants (e.g. cytokines and co-stimulatory molecules such as interleukin) (Toka *et al.*, 2004). ADP -ribosylating toxins have been used as effective mucosal adjuvants in higher vertebrates but have not yet been tested or established as mucosal adjuvants in fish.

A variety of treatments (hypo- and hyper-osmotic baths, scarring of skin surfaces, ultrasound irradiation, and combinations of hyper-osmotic baths and ultrasound irradiation) have been used in combination with anti-Gth immersion to enhance mucosal immune responses. These were referenced in the previous section.

CHAPTER 4

INTESTINAL IMMUNOLOGY TELEOST

In recent decades, mucosal immunology of higher vertebrates has been a heavily explored area of research. Although a well-functioning mucosal immune system in fish is highly beneficial for living in a pathogen-rich aquatic environment, few details are known about this system in this group of animals. This review summarizes the known data on the intestinal immune system of bony fish and compares it with the intestinal immune system of mammals, where appropriate.

In mammals, two sites can be distinguished in relation to the immune system in the intestinal mucosa: induction sites (GALT structures such as Peyer's patches) and effector sites: lamina propria (LP) and the intraepithelial lymphocyte compartment (IEL) [1,2]. M cells are very important in the follicle-associated epithelium above the induction sites. They can actively transport exogenous antigens into the underlying lymphoid tissue and ultimately lead to IgA class switching [3] and the secretion of large amounts of dimeric IgA at the effector. websites [1,2]. The secreted IgA is then bound by the polymeric Ig receptor (pIgR) and transcysted in the intestinal lumen or bile in the liver. The extracellular portion of the receptor is then cleaved off and secreted as a secretory component (SC) along with IgA (and IgM, both with a connecting J chain for pIgR binding) at the mucosal site. In the last decade it has been shown that dendritic cells (DCs) can also take up antigens directly from light and this new induction alternative is now well established.

Peyer's patches, M cells, IgA and J chain are not reported in teleosts. In addition, lymph nodes are absent and there is little evidence of specific mucosal lymphocyte homing. This almost rules out the existence of a common mucosal immune system, but does not rule out a local mucosal defense system. The first indications that fish could have local and/or mucosal reactions came from the detection of specific antibodies in mucosal secretions after intestinal [4e8] or immersion [9e11] immunizations of a large number of fish species, whereas they were then hardly or not at all detectable systemic immunization. vaccinations. This differential occurrence of specific antibodies combined with the detection of specific antibody-producing cells at mucosal sites after gut vaccination [12] or by immersion [11,13] has inspired many scientists to study mucosal structures in fish.

4.1. ABSORPTION OF FOOD AND ANTIGENS IN THE GUT OF FISH

The digestive tract of fish begins with a straight tube without the presence of a clear stomach. During larval development, the stomach develops in 85% of bony fish species [14], while the remaining 15% of fish species (ie, cyprinids) never develop a stomach and do not have a low pH pre-digestive region. In these gastric species, the bile and pancreatic ducts enter the intestines just behind the esophagus, and the first enlarged right part of the intestine, the intestinal bulb, appears to have a storage function. In almost all species studied, the gut can be divided into three segments based on the microscopic anatomy of their mucosa, particularly their enterocytes.

1) The first segment (60-75% of the total length of the intestine, depending on the species) whose enterocytes can be considered as absorptive cells. In functional studies of cyprinids [16-18], absorption of dietary protein appears to occur in the anterior 50% of the gut.

2) The second segment (15-30% of the length of the intestine), in which the enterocytes are characterized by large supranuclear vacuoles, an area of irregular microvilli and high pinocytotic activity in the apical part. In this part, a strong resorption of macromolecules such as horseradish peroxidase (HRP) and ferritin could be demonstrated both in species without a stomach [15, 18–21] and in species with a stomach [22–25].

3) The third segment (5-15% of the gut length) is less well studied, but the enterocytes are ascribed an osmoregulatory function and, due to the small microvilli, they do not appear to have a nutritional function [23, 26]. Much recent work neglects the third segment and mentions the rectum or hindgut when discussing the second segment of the intestine.

It should be noted that some species of fish (e.g. Gadidae) have a rectum in their hindgut, most often separated from the gut by valves; a second clear segment has not yet been found in cod [27]. The high absorptive capacity of the second segment has apparently no nutritional value and has attracted the attention of immunologists since the 1980s when it was shown that antigens can be transported to the local immune system as well as systemically [6,7,12, 28]. Crucial observations regarding antigen uptake and processing were made in carp [21], showing different transport pathways for receptor-mediated uptake (solid-phase uptake of HRP) and liquid-phase uptake (ferritin). HRP was immediately sorted into the endolysosomal compartment and sent to the intercellular spaces, while ferritin made its way to the large supranuclear vacuoles (SNV: endosomes) and could never be observed in the intercellular spaces. The soluble factors (mainly LPS) of a bacterin follow the same path [6,7,12]. The

macromolecules taken up by the liquid phase pathway are eventually transferred to phagocytes present in and beneath the intestinal epithelium, which are resident and motile macrophages in carp. Although the mechanism of this transmission is never well understood, the most logical route is the transmission of SNV from epithelial cells to phagocytes, as described for the transmission of melanin to human skin epithelial cells and the apical parts of rods to pagocytic chromatophores in the mammalian retina.. Receptor-mediated uptake of HRP leads to a very rapid HRP peak in the blood of carp[29] and trout[30] 30 minutes after oral administration, while the appearance of ferritin or LPS in macrophages is more of a 4-8 hour process [7,21]. The HRP receptor is currently being characterized, but the biological reason for the enzyme uptake mediated by this receptor is still unclear [31]. In contrast to mammalian M cells, second segment enterocytes have functional endolysosomal organelles and are not well able to phagocytize inactivated bacteria [32]. In contrast, phagocytosis cannot be completely ruled out and probably requires some inducing signals. For example, poly-D,L-lactide-co-glycolic acid (PLGA) microparticles appear to cross the intestinal barrier, enter the body and eventually end up in the systemic lymphatic system [33-35]. None of these articles showed the penetration mechanism of PLGA microparticles.

4.2 COMPETENT IMMUNE CELLS IN THE INTESTINAL MUCOSA

The presence of competent immune cells has been well studied in the gut of carp, European sea bass and Atlantic salmon. It has been known for many years that leukocytes are abundant in the lamina propria and in the intestinal epithelium [36-39]. However, the lack of appropriate antibodies in fish has hampered differentiation of subpopulations within the gut-associated lymphatic system (GALT). In bony fish, the degree of organization of the GALT is lower than in mammals, but they have a more diffusely organized immune system in their gut, which contains many lymphoid cells, macrophages, eosinophils, and neutrophils. To our knowledge, dendritic cells (DC) are not well established in the mucous membranes of fish.

4.2.1. Ig positive B cells

Igþ cells have been described in the intestinal mucosa of sea bass [40,41], salmonids (Sunyer, this edition) and cyprinids [39], but the reported numbers vary widely between species. In carp, the largest number of Igþ cells have been described, which are mainly present in the intestinal mucosa and consist of B lymphocytes and plasma cells, the latter being generally smaller than those found in systemic lymphoid organs [39]. The large difference in the number of B cells

found in this species may be due to the reactivity and/or affinity of the monoclonal antibodies used. As we shall see later (Section 5), mucosal Igs may differ from systemic Igs and antibodies against serum IgMs may not or hardly react with mucosal Igs. However, the monoclonal antibody against carp serum IgM (WCI12) seems to cross-react with mucosal Ig/B cells [42], and therefore it can be concluded from this species that B cells are abundant in the lamina propria. In isolated carp gut cell suspensions, the proportion of isolated B cells is low (5–10%) because the cells cannot be easily detached from the connective tissue [39]. Another possibility of low gut B cell counts in some species can be explained by a preference for mucosal plasma cells in the liver, as shown for an Antarctic species.

4.2.2. Ig negative T cells

Monoclonal antibodies (mAb) specific for T cells or T cell subpopulations have been used to detect these T cells in sea bass [40,41,44] and carp [45]. In both species, T cells were abundant in the lamina propria and epithelium. Intestinal epithelial lymphocytes (IEL) from a variety of teleost species have been shown to express T cell genes such as CD33, ZAP70, TCR, CD4 and CD8 [46-49]. An anti-human CD33 antibody raised against a well conserved recombinant peptide appeared to cross-react with various fish species and revealed an abundance of T cells in the epithelium and lamina propria of carp [50] and Atlantic salmon [51]. In sea bass, a significant number of DLT15þ cells do not express TCRb and this may be the first indication of the presence of gdT cells [52]. Preliminary results in bass and carp have shown gene expression of a gdTCR.

4.2.3. macrophages

Few studies have focused on fish intestinal macrophages, again probably due to the lack of suitable markers. Although intestinal macrophages have been morphologically described in a variety of bony fish species, functional evidence is only available in carp. Cyprinids have numerous macrophage-like cells in and under the intestinal epithelium and their number doubles after anal intubation with antigens. This invasion is mainly due to small motile macrophages that migrate and can be detected 1-2 days later in other lymphoid organs loaded with gut antigen. Many large resident intraepithelial macrophages can be found in the second gut segment of most of the cyprinids studied. These cells are strongly Igþ due to their Ig-binding capacity [39,54]. These macrophages can be loaded with antigens by light; after degradation, antigenic determinants can then be presented on their surface [6,53]. All of these

features and the abundant presence of B and T cells again strongly suggest that local immune responses can be elicited. It should be clarified here that not all bony fish species have a similar number and size of intestinal macrophages or do not have the same Ig-binding capacity.

4.2.4. granulocytes

In general, fish granulocytes can be considered as innate cells. Although intermediate cells are sometimes described as distinct subpopulations, two main types can be considered: neutrophils, which have the smallest granules with a typical rod-shaped electron-dense (EM) structure, and eosinophils, which have larger, electron-dense granules that closely resemble mammalian mast cells. These eosinophils are called basophils in common carp because they do not stain with eosin using standard fixation techniques. Reite decided to use the term mast cells/eosinophilic granule cells for the first time, but it must be said that fish have no IgE and, with the exception of one bearded fish, no histamine can be detected in these cells. On the other hand, they can release tryptase, antimicrobial peptides such as lysozyme, piscidin and pleurocidin, are abundant in the mucous membranes, and have a strong inflammatory response by migrating and releasing granules. Inflammation can be triggered by a variety of factors: diet, infectious agents, and chronic stress. In both fish and mammals, neutrophils are considered to be innate phagocytic leukocytes that are abundant in the circulation and are rapidly recruited from the blood at sites of inflammation. The "healthy" gut of fish generally contains fewer neutrophils than eosinophils/basophils, but they invade the epithelium more easily and their numbers increase sharply under conditions of danger or stress. Neutrophil-specific mAbs are available for salmonids and carp to study neutrophil behavior, but unfortunately they have little or no application for the teleost gut, apart from the recent thesis by Sundh; Both salmon neutrophils and eosinophils demonstrated a strong invasive and activating response to stress and infection, and neutrophils frequently invade the intestinal epithelium.

4.3. MUCUS IMMUNE REACTIONS AND ORAL TOLERANCE

As already mentioned, mucosal immunizations result in specific antibodies that are detectable in mucosal secretions, while these antibodies were resistant or undetectable after systemic immunizations [4e10]. These data were supported by the detection of plasma cells secreting specific antibodies (ELISPOT) in the gut and/or gills after mucosal immunization of carp and sea bass and their absence or rarity in the head, kidneys, spleen or blood. These results strongly indicate the induction of local reactions, but also that there must be differences between serum

and mucosal Ig. Some of the studies mentioned show that the antibodies detectable in the skin mucus appear relatively late compared to the appearance of serum antibodies after parenteral immunization. This could be due to a severe lag phase caused by the transport of bound specific antibodies into the multilayered skin epithelium, as plasma cell detection studies have shown comparable peak days after mucosal and systemic immunizations. In carp, oral immunization with encapsulated Vibrio anguillarum antigens (alginate) resulted in high numbers of specific plasma cells in the gills, which may indicate a common mucosal immune response. However, previously unpublished results in carp and more recent data in trout have not revealed a specific mucosal localization of IEL. Since IELs consist mainly of putative T cells, tissue-specific homing for teleost B cells cannot yet be ruled out. It has recently been suggested that "B cells need a proper home, while T cells are happy in a cave," indicating a higher B cell dependence on secondary lymphoid tissue " is B cells have yet to be detected.

Another characteristic phenomenon of gut immunology is oral tolerance or suppression of immune responses after (repeated) oral immunization. This phenomenon led to a decrease in the production of specific antibodies against bacterial and protein antigens in rainbow trout, salmon and carp. Only in carp has it been shown that oral protein administration can lead to a genetically determined suppression of antibody responses after systemic administration of the same antigen, which was comparable to oral tolerance studies in mammals. Tolerance induction for cytotoxic responses has also been described more recently when animals were repeatedly (anally or orally) immunized with allogeneic target cells (EPCs). Interestingly, this suppression of cytotoxicity caused by repeated intestinal administration was restored when the same antigen was administered systemically. None of the oral safety studies have addressed the mechanism behind this phenomenon. It is also unclear how orally or anally intubated whole allogeneic target cells can overcome the intestinal barrier and drive specific cytotoxic cells into the blood.

4.4 MUSCLE IMMUNOGLOBULIN

The pronounced secretion of antibodies in mucus can only be explained by differences in mucosal and systemic immunoglobulin (Ig). But unlike higher vertebrates, IgA is undetectable in fish. The biochemical analysis of skin mucus of different bony fish species revealed only IgM-like molecules, only in tetrameric form, but also in dimeric and monomeric form. Currently there is little data on the presence of Ig in the intestinal mucus, probably due to the difficult access, but also due to the degradation of IgM in the highly proteolytic environment.

Along with IgM, IgD is an immunoglobulin commonly found in teleosts, but so far this isotype has never been correlated with mucosal immunity. Although differences between serum and mucus Ig were observed in sheep and carp, no molecular evidence for the different IgM subtypes was found. In carp, a mAb could be selected that reacted with mucus H chain and not with serum IgM. This mAb could be used to detect specific plasma cells in the gut and gills after mucosal immunization and was immunoreactive with bile ducts and capillaries in the liver and with skin epithelial cells, only sites where mucosal Ig can be expected. A non-covalent dimer of covalent dimeric subunits associated with a 95 kDa secretory component-like protein has been described in the sheep's head (Archosargus probatocephalus), but this has not been found in the bile of the same species and has never been observed in other species. A J-chain is never found in teleosts, while this IgA or IgM-binding protein is present in all vertebrates, including elasmobranchs. Nevertheless, it can be concluded that teleosts have a mucosal immune system because intravenous administration of radiolabeled Ig has never reached the mucosal secretions [84] and therefore the Ig in the mucosal secretions must be the result of local synthesis. To date, the liver and bile have been little studied, but the presence of Ig in the bile and the accumulation of plasma cells in the hepatic portal tracts of an Antarctic teleost fish, Trematomus bernacchii, indicate that the liver, as in mammals, may have a be an important secretory site. for mucosal Ig. In 2005, a new, previously unknown Ig isotype was described, which is called IgZ in zebrafish and carp, IgT in trout and IgH in fugu. Recently it has been claimed that IgT in trout is specialized in mucosal immunity and that IgT responses to an intestinal parasite are restricted to the gut. In carp, two subclasses of IgZ are described (IgZ1 and IgZ2), of which IgZ2 is a two constant domain chimera with m1 and z4 domains. IgZ1 is more abundant in systemic organs, while IgZ2 is preferentially expressed in gills and intestines. Indeed, IgT or IgZ can be an important last flag up [86], but on the other hand, not all teleost species seem to have the IgT or IgZ-like sequences like channel catfish, so this species showed mucosal reactions.

4.5. THE POLYMERIC IG RECEPTOR (PIGR)

pIgR is an essential component of mucosal immunity in mammals. It is expressed by mucosal epithelia and hepatocytes, can bind IgA and IgM, transcylate these Ig to luminal (or biliary) sites, and finally the extracellular part of the receptor is cleaved off and co-secreted with IgA or IgM as a protective secretion. component [1,2]. In mammals it contains five Ig-like domains (ILDs), but only four ILDs are found in birds [93] and amphibians [94]. Recently, the pIgR

amino acid sequence of four teleosts (fugu [95]; carp [96]; orange-spotted grouper [97]; rainbow trout [90]) and two others (zebrafish and Atlantic salmon) reported by GenBank has been published. The teleost pIgR consists of only two ILDs corresponding to mammalian ILD1 and ILD5 [95-97]. It is evident that the CDRs required for IgA binding on ILD1 in teleosts are missing all three. However, IgM binding studies have shown that this low molecular weight pIgR can bind to teleost IgM and to cells that strongly express the pIgR: dermal epithelial cells, enterocytes and hepatocytes. The binding of pIgR to IgM in mammals is associated with the presence of a J-chain, which is why it has been postulated [95,97] that the J-chain must be present in teleosts as in elasidates. However, it is also suspected that the mammalian J chain does not interact directly with the pIgR but simply creates the conformational site necessary for binding with the pIgR [83]. On the other hand, mammalian, avian, and amphibian J chain is able to polymerize human IgA while nurse shark J chain is not, probably because of the highly conserved J chain region for pIgR interaction in Tetrapods do not lack cartilaginous IgA. fish [94]. Therefore, one can still wonder to what extent the pIgR and IgM interaction occurs in teleosts without the presence of a J chain. Recently, the interaction of pIgR and IgT in trout was demonstrated [90]. Another point of debate is whether the low molecular weight (35 kDa) pIgR will be cleaved as a secretory component (SC) after transcytosis and whether it will differ between polarized epithelial cells or hepatocytes and epithelial cells. With the exception of sheepshead [82], SC is never described in mucosal IgM isolated from other species. In addition, the molecular weight (95 kDa) of this SC is far too large to be linked to teleost pIgR. Theoretically, skin epithelia can be well protected if Ig binds to the surface of these cells. Transport of this bound Ig may explain the aforementioned lag phase observed in cutaneous antibody responses following mucosal immunization.

4.6. INTESTINAL T CELLS AND THEIR FUNCTION

Mucosal T cells represent the major leukocyte population in the teleost gut, but limited data are available on their functional relevance. Rainbow trout ab-IELs show marked changes in the TCRb repertoire after systemic rhabdovirus infection, comparable to spleen and pronephros T cells. Recent data on sea bass (Dicentrarchus labrax) have led to a better understanding of their local defensive functions [46]. Gene transcription studies revealed the large presence of T cells in the gut and TCR-b and CD8-a transcripts exceeding those of CD4, indicating the predominant presence of CD8-a expressing T cells. In particular, the CD8-a transcripts increased significantly in the posterior part of the intestine. These data are consistent with

previous findings of a significant increase in mAb DLT15þ T mucosal cells along the gut from the bass to the anus, which largely depends on the increase in DLT15þ IEL in the posterior segment. This reflects regional immune specialization as previously reported in other bony fish species. In situ hybridization studies with digoxigenin-labeled RNA probes strongly confirmed the Q-PCR data, indeed numerous cells expressing TCR-b and CD8-a were identified in the posterior gut mucosa of the beam, whereas only rarely CD4-expressing ones Cells and very rare MHCII-b expressing cells were located. Indeed, MHCII-b transcripts were detected at even lower levels in the posterior than in the anterior and midgut, while MHCI-a transcript levels were independent of CD8 transcript fluctuations along the gut. This suggests that a subset of CD8-aþ T cells might bind antigens outside of the context of MHC molecules, as demonstrated for the mammalian TCR-gd CD8-aaþ IEL subset that does not require presentation of the peptide that is restricted to the MHC or becomes positive selection.

Lymphocytes purified from sea bass intestinal mucosa showed significant cytotoxic activity against xenogeneic (K562) or allogeneic (DLEC) target cells [46]. Such an antibody and complement (serum-free medium) independent response was indicative of the presence of non-specific, MHC-independent activity. Teleost lymphocytes (sIgM, CD8þ) show specific activity against allogeneic targets but also cytotoxicity if they are not sensitized. Indeed, IELs isolated from rainbow trout gut were spontaneously cytotoxic against a mouse tumor cell line. Unfortunately, little information is available on the regulatory mechanisms involved in immune homeostasis in the gut of teleosts. WCL38-sorted carp IELs (mAb specific for mucosal T cells) have no non-specific cytotoxicity but can express IL-1b, IL-10, TNF-a and TGF-b (unpublished). IEL of the inflamed second intestinal segment of carp showed an upregulation of proinflammatory cytokines (IL-1b, TNF-a) and an initial upregulation of IL-10 and later in the enteritis process an upregulation of TGFb. In rainbow trout, expression of IL-1b, TNF-a, IFN-g, IL-8 and TGF-b was found in tissue samples from the distal or proximal gut; after the Aeromonas Salmonicida dip, most genes were upregulated in the proximal gut tissues, but TGF-b was downregulated in the distal gut, where it had the highest basal expression. Given these results, a regulatory role for at least one teleost IEL subpopulation is likely.

In mammals, intestinal gdT cells have been proposed to play an active, multifaceted regulatory role in coordinated innate and adaptive immune responses that maintain epithelial tissue integrity. They induce cytolysis of infected or transformed intestinal epithelial cells, support mucosal IgA production, maintain epithelial homeostasis via an intranet with abT cells and

epithelial cells, and may play important regulatory roles in inducing oral tolerance, gut metabolism, and preventing unwanted play immune responses.

4.7 ONTOGENES OF THE INTESTINAL IMMUNE SYSTEM

Reports on the development of the immune system in fish are numerous and most of them are devoted to zebrafish. Like mammals, zebrafish have distinct waves of hematopoiesis [118,119]. The first wave appears in the intermediate cell mass for erythroblasts and in the anterior mesoderm (also called the rostral blood island) and produces primitive myeloid progenitors that migrate to the yolk sac and differentiate into primitive but functional macrophages [22 hours after fertilization (hpf); [120]] and the production of primitive neutrophils [at 33 hpf; [121]]. Multiline hematopoiesis begins at about 24 hpf with the formation of a transient population of erythromyeloid progenitors in the posterior island of blood (around the cardinal vein just caudal to the anal region), which later develops into the caudal hematopoietic tissue. This analogue of the mammalian liver produces erythroid and myeloid cells. These cells migrate again in the 3rd dpf and begin to colonize the final hematopoietic tissues: (head) kidney and thymus. In zebrafish, the spleen is not considered a hematopoietic site, but in other species, such as carp, the spleen is the major erythropoietic and thrombopoietic organ. Data on the ontogenesis of the intestinal immune system in fish are rather sparse. Although two recent papers have provided a detailed description of the early development of the zebrafish gut [124,125], no attention has been paid to the development of the GALT. The limited data available on the development of GALT in fish are summarized here.

4.7.1. myeloid cells

Myeloid progenitors differentiate into monocytes/macrophages, neutrophils, and eosinophils/basophils. From 2 dpf (zebrafish) and 3 dpf (carp) the posterior blood island must be considered as the main production site of myeloid cells. In carp, a macrophage-specific mAb was used to show the first appearance of macrophages in the renal region of the head at 2 dpf and in the spleen and intestine at 4 dpf and in the thymus at 7 dpf. Macrophages can be observed within the intestinal epithelium from as little as 4 dpf, and their number and size increase during ontogeny.

Carp neutrophils and eosinophils/basophils can be isolated from embryos as early as 2 dpf, but their numbers increase at 5 dpf when the posterior island of blood has expanded significantly. However, data on the occurrence of neutrophils and eosinophils/basophils in the gut are scarce

or absent. Using a carp neutrophil-specific mAb, it was shown that the greatest number of neutrophils could be isolated from the gut (10-15% of the leukocytes isolated) at 10 dpf, but the proportion of these granulocytes gradually decreased at later time points. In gnotobiotic zebrafish, an influx of intestinal neutrophils and upregulation of the neutrophil biomarker myeloperoxidase were observed in response to microbiota at 8 dpf, indicating that both commensal and pathogenic microbes can upregulate immune cell activity at these early stages. Nothing is currently known about the presence and function of eosinophils/basophils in the gut of developing fish.

4.7.2. Ig negative (T) cells

In carp and zebrafish, the thymus is populated with lymphoid cells between 3 and 4 dpf [118,133]. In carp gut, Rag-1 expression can be detected from 4 dpf and in situ hybridization revealed single rag-1þ cells around the basement membrane from 7 dpf. The first immunoreactive T cells could be detected in the intestinal epithelium at 3 dpf with a mAb (WCL38, reactive with mucosal T cells) and their number increased sharply within a week [129]. Most, if not all, WCL38þ cells are CD33þ cells, and CD33 expression is detected in carp gut from 3 dpf, while ZAP70 expression occurs in gut at 7e10 dpf versus 5 dpf in thymus (preliminary results). Although the precise nature and function of early intestinal WCL38þ cells is not yet known, it cannot be excluded that this putative T cell population develops and differentiates without thymic interference. The existence of an extrathymic pathway for IEL generation has also been suggested for mammals [134-136], although the opposite is also debated. TCR-gd CD8-aaþ IEL in mammals can develop in the absence of a functioning thymus gland. Although the origin of intestinal T cells in teleosts is still unknown, Q-PCR analysis showed that rag-1 transcript levels were significantly correlated with those of CD8-a in different intestinal segments of single-bar samples. The sea bass gut could therefore be considered a site of somatic rearrangement, as previously demonstrated in mammals [140] and proposed in other bony fish species such as zebrafish [141] and carp [129]. In any case, more research on the ontogenesis and expression of T cell molecules/genes in fish could support this ongoing debate, especially now that the TCR-gd and CD8-a genes can be detected in fish [cf. Castro et al. in this problem].

4.7.3. Ig positive B cells

Lobe-dependent rearrangements of B-cells in the pancreas have been described in zebrafish, but this could not be confirmed during ontogeny in carp. In this species, the head kidney appears to be the primary candidate for B-cell differentiation, since Rag-1, Rag-2, and early IgH transcripts are found in this organ at 7 dpf. The first IgM immunoreactive B cells are detected around 2 wpf and the first plasma cells at 4 wpf. In the gut and gills, the first B cells of carp appear in the 6th wpf, and this period of "lag" between the appearance of B cells in systemic versus mucosal organs is also found in perch and spotted wolf. It has recently been shown that IgZs in carp do not appear earlier in ontogeny than IgMs. The rather late appearance of B cells in the mucosal tissue of the species mentioned suggests that adaptive mucosal immunity appears to develop later than systemic immunity, while IEL appeared much earlier (3 dpf). At present it cannot be ruled out that early carp IELs mainly have innate immune functions.

4.8. ORAL VACCINATION STRATEGIES

Vaccination of fish has a long history, but only in the last 20 years has vaccination against bacterial and, more recently, viral diseases become established as an effective prophylactic treatment. Vaccination of fish by injection or immersion is now widely used, and some oral vaccines have recently come onto the market. This contribution limits oral vaccination as the most interesting method, since it is easy to administer without causing stress and is suitable for mass vaccination at any age. Interestingly, the first paper on oral vaccination was published by Duff in 1942, showing that trout could be orally vaccinated against Bacterium Salmonicida; prolonged feeding (64-70 days) of a diet containing B. salmonicida resulted in 25% mortality after immersion challenge compared to 75% in control animals. In the 1980s, many trials of oral vaccination were reported, but most did not or did not reach these protective levels. Johnson and Amend (1983) showed very good protection after anal intubation with the bacteria V. anguillarum and Yersinia ruckeri compared to oral intubation or immersion. Therefore, it was concluded that oral vaccines should be protected from degradation and should be as inexpensive as possible. The first attempts in this direction were based on the bioencapsulation of V. anguillarum bacteria in young sea bream and carp using plankton as water flea, rotifers or Artemia nauplii as transport vehicles. The results also showed that this approach must be started at the right age, because animals that are too young still develop a kind of “neonatal” tolerance (immunosuppression). Other attempts to protect antigens from degradation in the gut include the use of coated bacteria (Eudragit), microbial biofilms [and more recently, bacterial

ghosts. A variety of microparticles have been tested and most studies report better rectal clearance, higher antibody production and/or protection using alginate microparticles, liposomes, poly-D,L-lactide-co-glycolic acid (PLGA) and chitosan -microparticles. Alginate particles, formulated in a manner tailored to the properties of the digestive tract, showed promise in carp and trout, eliciting mucosal and systemic immune responses. PLGA and chitosan particles are claimed to be biodegradable mucoadhesive microspheres, both of which are very suitable for encapsulation of pDNA vaccines. Although microencapsulation has been shown to be effective for oral vaccination of fish, there are few oral vaccines on the market, which may be due to the stability of the vaccine in the diet and the limited protection obtained, but is more likely due to the cost-effectiveness of these vaccines.. From this point of view, the development of "edible vaccines" for aquaculture can be promising. For example, viral trimeric G proteins can be produced in potato tubers (or other edible plants) and then used to make fish feed. When LTB (the non-toxic pentameric variant of the cholera toxin) is combined with Protein G, a very stable LTB-G protein construct is produced by plants and ultimately results in much better second segment uptake and immunological memory formation. The main problem currently to be solved with this approach is to increase the amount of transgenic proteins produced by the plants in order to avoid an additional concentration step. In fact, LTB can be considered as a mucosal adjuvant. In general, gut vaccination is hampered by the lack of appropriate mucosal adjuvants, and further research is also needed in fish to achieve optimal effects with low levels of antigen.

4.9 INTESTINAL MICROBIOTA AND PROBIOTIC IMMUNISTULATION

The gut microecology of fish is a relatively understudied aspect, but an impressive body of data on the use of probiotics has accumulated in recent years. The microbial community in the gut of fish is strongly influenced by the environment and within this complex ecosystem, microbes compete for space and nutrients for survival. Bacteria (107–108 per g) are the dominant microorganisms in fish gut, of which Proteobacteria are the most important phylogenetic type [167–170], while in mice and humans 99% of gut bacteria are Firmicutes and Bacteroidetes. Resident microbes, occasional pathogens, as well as artificially administered microbes (probiotics) can play an important role in the gut's immune response. They coexist in a dynamic equilibrium within the gut through continuous and active signaling. This symbiotic microbial consortium begins to develop with the first feeding in the larval stage and finally reaches a stable composition in the juvenile stage after autogenous and allogeneic

succession. Changes in the composition of the microbiota are very influential during the early stages of fish when gut colonization and immune system development take place. For example, the lack of microbiota during the developmental stages of zebrafish resulted in differentiation arrest and impaired function. As in mammals, the interactions between the host and commensal bacteria and the benefits derived from these processes are poorly understood. In either case, the innate immune system protects the host by maintaining the integrity of the gut barrier using pathogen recognition receptors (such as the TLR). For example, the generic microbial molecule LPS could serve as a ligand for TLR and regulate alkaline phosphatase, a marker of enterocyte maturation and lipid uptake. The beneficial microbial consortium uses multiple independent signals to interact with the host and coexist with pathogenic bacteria. It has been suggested that an imbalance in the microbiota that favors less traditional cultivars (referred to as "dysbiosis") could potentially lead to disadvantages related to the host's immune system, including an imbalance between regulatory T cells and effectors and a increased differentiation. inflammatory effector cells of the immune system. Microarray analysis of gnotobiotic zebrafish that received early gut bacteria revealed transcriptional changes resembling those of mice, thus indicating a conserved evolutionary role of the microbiota in vertebrate development. The innate immune genes involved were serum amyloid A1 (Saa1), C-reactive protein (Crp), complement component 3 (C3), angiogenin 4 (Ang4, a microbiota-regulated antibiotic protein) suppressor of cytokine signaling 3 (Socs3), myeloperoxidase (Mpo; a specific marker for granulocytes) and the oxidative stress response gene glutathione peroxidase 2 (Gpx2). In zebrafish, only fish microbiota (dominated by Aeromonas and Pseudomonas spp., alongside Vibrio and Lactococcus) and not those transplanted from mice showed increased expression of the innate immune response biomarkers e Saa, complement factor b (Cf), and Mpo. In addition, Saa and Cf responded to a large number of bacterial strains tested, while the granulocyte marker Mpo was specifically expressed with Pseudomonas aeruginosa. Although not all reports on the subject in fish are mentioned here, it is clear that host microbiological commensalism as well as the etiology of pathogenic conditions in the gut of fish require much more attention, especially as the application of probiotics appears to be a powerful tool. Fish disease resistance tool. Probiotics, currently defined as "live microorganisms which, when administered in appropriate amounts, confer a health benefit on the host" are considered a viable alternative therapy and have become an integral part of medical practices in aquaculture to enhance growth and disease resistance. Effects have been documented in protecting against a variety of bacterial diseases, but more recently against protozoal and viral infections: Ich in Oncorhynchus mykiss and Iridovirus in Epinephelus coioides. Protection against pathogens

has been claimed through competitive exclusion of adhesion sites, production of organic acids, hydrogen peroxide and other compounds such as antimicrobials, siderophores and lysozyme, and modulation of fish physiology and immunity. However, the mechanisms by which probiotics effectively modulate the immune system are not well understood, and the observed effects on local intestinal immunity are even rarer [183-185]. Viable probiotics (Lactobacillus rhamnosus) appear to be better immune stimulators than killed bacteria in rainbow trout, possibly due to their ability to colonize the host's gut. Recent studies in seabream and European sea bass for early administration (during gut metamorphosis) of autochthonous bacterial strains have shown profound effects on gut microflora, gut physiology and immunity of fish larvae. In sea bream, a probiotic multispecies formulation (native Lactobacillus fructivorans þ Lactobacillus plantarum from human feces) increased the number of intestinal IgMþ cells and acidophilic granulocytes [190]; This formulation was significantly more effective (in terms of juvenile survival and improved stress response) when administered early. However, juvenile sea bream fed diets containing Lactobacillus delbreckii ssp. lactis and Bacillus subtilis (individually or combined) also showed higher total serum IgM and intestinal IgM² and acidophilic granulocyte counts than untreated controls. In sea bass, the autochthonous bacterium Lactobacillus delbrueckii delbrueckii increased the number of intestinal T cells (with an increase in whole body TcR-b transcripts) and acidophilic granulocytes, along with decreased transcription of important inflammatory genes (IL-1b, Cox-2, IL-10, TGF-b). Despite this down-regulation of cytokine expression, increased survival, increased weight gain, decreased cortisol levels, and improved stress responses were observed. In Atlantic cod, autochthonous gut bacteria also appeared to be potential probionts, and proteomic analysis of probiotic-fortified cod showed lower levels of immune stimulation but upregulation of proteins involved in growth and development. It is clear that the data provided on probiotic stimulation is fragmentary and likely dependent on bacterial strains or fish species and therefore much more research is needed to explain the reported protection after probiotic administration.

4.10. PATTERNS OF ENTERITIS IN FISH

In the last decade some interesting patterns of intestinal inflammation have been described and studied in more detail. Replacing fishmeal with soybean meal (SBM) resulted in inflammation of the hindgut of Atlantic salmon [53,195,196], and the severity depended on the amount and variety of soy used. The animals showed normal growth and feeding, but developed severe inflammation of the second segment of the intestine, characterized by: the disappearance of the

supranuclear vacuoles (SNV) in the enterocytes of the second segment, a sharp decrease in the height of the microvilli, swelling of the lamina propria and the subepithelial mucosa, an increase in the number of goblet cells, a strong invasion of eosinophilic granulocytes, a loss of architecture of the mucosal folds and finally a complete tissue rupture and when feeding SBM is continued for 4 months, chronic intestinal inflammation even led to the development of adenocarcinoma with liver metastases. Recently it has been described that the beginning of the whole process is a complete blockage of endocytosis in the second intestinal segment, which well explains the disappearance of the SNV, the endolysosomal compartment of these enterocytes. In addition, the enzymatic profiles of intestinal epithelial cells are greatly altered. Soy saponins have been shown to be responsible for the inflammatory response in combination with one or more as yet unidentified dietary components, since the saponins alone only increase intestinal permeability. As in mammals, two proteinase-activated receptors (PAR-2a and PAR-2b) play important roles in the inflammatory process. During the inflammatory process, changes in various immunological processes are monitored: a marked increase in neutrophils and eosinophils in the lamina propria, degranulation of eosinophils, increased IgM staining of LP without change in IgM cells þ and an increase in T cell responses for invasion of CD3-3 þ cells and increased expression of CD3-pp, CD4, CD8-a and CD8-b [49]. The severity of SBM-induced enteritis differs between species. Less severe enteritis is observed in rainbow trout and no pathological development has been reported in several other species (Atlantic halibut, Atlantic cod, channel catfish, Nile sole) [cf. 50]. Juvenile cobia (Rachycentron canadum) also lacked clinical signs when using a similar SBM. In all cases, however, histopathology was performed 6–9 weeks after the first feeding. In carp, a similar process of SBM-induced inflammation was described during the first four weeks after feed change, but by week 5, carp begin to fully recover from SBM-induced enteritis without changing the regimen. Similar immunological reactions were observed in carp during the enteritis process: invasion and degranulation of granulocytes, higher activity of T-lymphocytes, but also up-regulation of pro-inflammatory genes IL-1b and TNF-α1 and down-regulation of anti-inflammatory drugs. IL-10. TGF-b appears to be upregulated in carp 3 weeks after SBM feeding. In Atlantic salmon, TGF-b, IL-1b, interferon-g-inducible lysosomal thiol reductase (GILT), but also CD3 and CD8-b were downregulated during the first week of SBM-induced enteritis. It has been suggested that SBM-induced enteritis in salmon may be related to down-regulation of TGF-b, a potent control of immune homeostasis and mucosal inflammation. The upregulation of TGF-b after 3 weeks in carp while remaining downregulated in salmon may support the important role of TGF-b in this enteritis model. A new enterocolitis model using anal intubation with

oxazolone has been developed in zebrafish. A partially similar course of enteritis is described: epithelial damage, thickening of the intestinal wall, loss of the architecture of the intestinal folds, influx of granulocytes and increased expression of IL-1b and TNF-α1. In contrast to carp and salmon, a decrease in goblet cells was observed but an increase in IL-10 was reported. In zebrafish, the severity and susceptibility of oxazolone-induced enteritis can be influenced by changes in the composition of the gut microbiota. Therefore, zebrafish is considered a good model to study the microbiota/enteritis relationship, especially since zebrafish can be produced aseptically.

CHAPTER 5

BACTERIOLOGY

Small fish should be preserved alive or fresh dead (within 1-2 hours) on blue ice in one Cooler. fish homework not to be frozen. bagged fish should not to be in direct Contact with blue ice cream Where fromher or her will Freeze.

Life fish are to prefer to the diagnostic Rehearse. AT fewer 5-10 dying fish should to be setin one or more large leak-proof plastic bags with breeding water. Seal the bags with it Space remains for air and leaks do not occur. Label the bags with the condition of the fish (dying vs healthy), incubator Where from path of the cables Number, camp and species and join a Taste templateForm (see side 1-8) with each Shipment. Yes oxygen east available, add to the bags In front Seal. additive from a oxygen tablet to the each bag east devices, especially to the rehearse The homework to be Sent.

Bacterial diseases are responsible for the high mortality in wild and farmed fish. However, most of the microorganisms responsible are saprophytes of natural origin, which play an important role in the synthetic pathways and degradation processes of the aquatic environment, using the organic and mineral matter of the environment for their growth and reproduction. Several researchers have shown that the normal bacterial flora of fish is a direct reflection of the bacterial population of the water in which they swim (Horsley 1973; Sakoto et al. 1980; Kim et al. 2006). These microorganisms are essentially opportunistic pathogens that invade the tissues of a host fish rendered susceptible to infection by stressors or other pathological processes. The most important group of microorganisms in this regard are motile aeromonads (Allen et al. 1983; Austin & Austin 2007), although other gram-negative bacteria, as in higher vertebrates, probably play an important role, but have not yet been well studied. However, a few bacterial species appear to be obligate parasites of fish. Although they can survive in water for varying lengths of time, they do not appear to reproduce significantly outside of the host. But diseases caused by these primary pathogens are almost always stress-related. Latently infected but clinically healthy fish do not usually succumb if the environmental conditions remain favorable, but can be long-term carriers of the pathogen and infect other fish, especially under stress. Usually, the clinical disease manifests only after a major change in the fish's physiology due to exposure to an external stressor, or occasionally in conjunction with an internal change such as spawning.

Research into bacterial fish diseases is hampered by the current lack of adequate understanding of the interactions between bacteria, their hosts and the aquatic ecosystem in general. Communicable fish diseases occur when a susceptible host and a virulent pathogen come together in an environment conducive to such occurrence. Some bacterial pathogens such as Vibrio anguillarum or Aeromonas Salmonicida are robust primary pathogens that require little from their environment or host to allow overt infection. Others are less able to induce infection and produce clinical disease when present in overwhelming doses or in fish affected by their internal or external environment.

Although there is now a greater recognition of the need to study bacterial diseases in the context of the host and environment, and these conditions are almost always related to stress, the exact changes that trigger susceptibility to invasion are not known. They are likely related to suppression of primarily non-specific defense mechanisms such as the reticuloendothelial system, changes in the integrity or physiology of mucous surfaces, or degradation of heat shock proteins - chaperone-producing genes (Roberts 1993; Roberts et al. 2010). Regardless of the nature of these increased susceptibility mechanisms, when fish resistance is reduced, microorganisms will invariably be available to exploit. They may already be present in some or all fish in the population, whether in the tissues, gut or external environment, and can invade and cause clinical disease.

Aquatic environments with high organic loads that support bacterial growth, rapid temperature changes, overcrowding, trauma, and transport are the most commonly encountered environmental stressors that predispose to clinical disease in fish. These factors are particularly likely to occur in intensive fish farming systems.

The rapid global expansion of aquaculture in recent years has led to a corresponding predictable increase in the incidence and severity of long-established bacterial diseases and the emergence of a number of new infectious diseases. Environmental stress should be carefully considered when assessing the importance of bacteria isolated from diseased fish, since chemotherapeutic treatments or prophylactic vaccination methods rarely produce a fully satisfactory clinical cure unless measures are also taken to correct the mediating factors.

The invasiveness of a microorganism is clearly one of the most important aspects of its pathogenicity. Certain species such as Aeromonas Salmonicida (McCarthy & Roberts 1980) or Mycobacterium marinum (Aronson 1926) are generally considered to be primary pathogens

and as such are capable of causing severe disease in most fish species with only limited stress. Others such as Aeromonas sobria or Aeromonas hydrophila (Thorpe & Roberts 1972) can only penetrate fish tissue that is already heavily stressed. There is also a third group of bacteria associated with diseased fish. These are practically saprophytic but invade the tissue of fish perimortem. They can play a role in the eventual death of fish, but they cannot be considered pathogenic. They also rapidly multiply in tissues after death, so they can be easily isolated from such individuals. For this reason, the validity of bacterial isolates from dying or dead fish at the time of sampling must be interpreted with great care.

5.1 TAXONOMY

All taxonomic systems have an element of aesthetic unreasonableness (Cowan 1955), and nowhere is this more apparent than in the pathogenic bacteria of fish, since they are individual members of distinct groups and pathogenicity is often the determining factor for teleosts. only trait they share. Although there can be many closely related non-pathogenic species in the aquatic environment, no detailed classification of these species or their families exists. This is largely because the strong economic incentive that drove the definition of fish pathogens and fish spoilage bacteria was not in place to produce a similar effect for these economically less important ones.

Representatives of about 92 bacterial genera have been implicated as pathogens of freshwater and/or marine fish (Austin & Allen - Austin 1999). However, as methods for identifying bacteria improve and classification systems are updated, the number of genera and species within each genus that are referred to as pathogens is constantly changing. Some notable examples of such changes include the reclassification of Haemophilus piscium as an atypical Aeromonas Salmonicida, the creation of a new genus, Renibacterium, for the bacterial organism of kidney disease, and the use of the Flavobacterium genus to encompass previously associated slippery bacteria chondrococcus or cytophages. The highly pathogenic species Vibrio anguillarum was renamed Listonella anguillara by MacDonnell and Colwell in 1985. However, this change has not been widely accepted, and although some reports use this name, Vibrio anguillarum is still the usual designation.

The majority of fish pathogens are gram-negative short rods belonging to the families Enterobacteriaceae, Aeromonadaceae, Pseudomonadaceae or Vibrionaceae. Generally they cause septic and ulcerative conditions. Long-hatched Gram-negative bacteria of the

Flavobacteriaceae family, which are not normally recognized as pathogens in warm-blooded animals, can also cause high mortality in fish stocks. Gram-positive microorganisms, some of which are acid-fast, are less commonly encountered but under certain conditions can cause severe losses in certain fish species. For example, members of the Streptococcaceae, previously thought to be of minimal concern, are now recognized as important pathogens under some of the newer, more intense conditions used for breeding fish species such as tilapia.

5.2 BACTERIAL DRUG RESISTANCE

Antimicrobial substances are now widely used to treat bacterial fish diseases and, more importantly in relation to bacterial drug resistance, are sometimes administered to prevent the development of diseases following the imposition of environmental stresses caused by cultivation methods be able. such as storage or transport. Drug resistance can be natural or acquired. For example, gram-negative bacteria are inherently resistant to penicillin and bacitracin because these antibiotics work by blocking the synthesis of cell wall components found only in gram-positive organisms. However, most antibiotics work by interfering with the function of cellular ribosomes, and resistance to these agents can be acquired either by mutating a chromosomal gene that changes the structure of the ribosomal target, or by infecting the cell with a resistance factor-R plasmid..

Plasmids are extrachromosomal circular DNA molecules capable of autonomous replication. They have a wide host range and are often easily transmissible between different bacterial species. R-factor plasmids carry genes that code for enzymes that catalyze the conversion of antibiotics into inactive derivatives. These plasmids often confer resistance to multiple antibiotics simultaneously, leading to the establishment of multidrug-resistant bacterial strains.

Evidence of transmission of the R factor to fish pathogens was first reported by Aoki et al. (1971) who showed that certain strains of Aeromonas Salmonicida carried R factors conferring resistance to a range of antibiotics and chemotherapy. The same research group has since reported the presence of transmissible R-factor plasmids in drug-resistant strains of Aeromonas hydrophila, Vibrio anguillarum, Vibrio sp. marin, Edwardsiella tarda and Pasteurella piscicida. This horizontal transfer of mobile genetic units has played an important role in the spread of antibiotic resistance. This widespread genetic exchange between bacteria of human, animal, fish, and environmental origin is ancient, as Sun et al. (2009) in a study on the genetic

mechanisms of multi-antibiotic resistance in a pathogenic strain Edwardsiella tarda. This has clearly demonstrated the importance of careful management of the use of antibiotics in the aquatic environment, whether released by humans or animals, or used on fish as such.

Continued use of antibiotics at sub-therapeutic levels to prevent disease increases the likelihood that populations of multidrug-resistant strains of pathogenic bacteria will build up. These can eventually lead to outbreaks that cannot be controlled with antibiotic therapy. This is already beginning to happen in several parts of the world. In addition, the presence of R-factor-infected bacterial populations in aquaculture systems leads to the transmission of antimicrobial resistance (resistance to infectious drugs) to other microorganisms, including potential human pathogens.

5.3 DIAGNOSTIC TECHNIQUES IN FISH BACTERIOLOGY

A description of the techniques for isolating and identifying bacterial pathogens from fish.

5.3.1 Fish Pathogenic Bacteria

FLAVOBACTERIACEAE

Fish pathogens now assigned to the genus Flavobacterium within this family were previously assigned to different groups including Chondrococcus and Cytophaga. They are all rod-shaped gram-negative bacteria that typically have an unusual gliding motion on solid surfaces. Collectively, they are still often referred to as "myxobacteria" due to their frequent association with mucoid surfaces. All members of the Flavobacteriaceae grow best on nutrient-poor media. Their colonies are often pigmented yellow-green or brown. They have a chemo-organotrophic metabolism and are generally aerobic. They can normally degrade gelatin and chitin and are usually positive for oxidase, phosphatase and ribonuclease, but biochemical or serological methods of distinguishing the species are not yet sufficiently advanced for clinical use, so diagnosis is largely based on clinical effect.

5.4 BIOCHEMICAL CHARACTERISTICS OF COMMONFISHBACTERIAL pathogens

A. *Aeromonas Salmonicida:* “Gram-negative rod, immobile, tan on TSA, cytochrome Oxidase positive, fermented O/F glucose, 2,3-butanediol negative, 0/129 resistant. Seeappendix A to the exceptions.”

B. *Aeromonas hydrophila:* "Gram negative rod, motile, cytochrome oxidase positive, fermented FROM glucose with natural gas, 0/129 and Novobiocin resistant, Vosges Proskauer positive. Just organization in the Water-Surroundings The east 2,3-butanediol positive."

C. *Aeromonas* sp. except *A. hydrophila:* "Gram-negative rod, variable motility, 2.3- Butanediol negative, cytochrome oxidase positive, fermented O/F glucose, 0/129 u Novobiocin resistant."

D. *Pseudomonas fluorescens:* "Gram negative rod, motile, cytochrome oxidase positive, FROM oxidizer, idle Where from alkaline. product fluorescein Pigment. Made not grow at 42 degrees."

E. *Vibrio ordal:* "Gram negative rod, motile, cytochrome oxidase positive, fermented O/F Glucose, 0/129 and Novobiocin sensitive, lysine decarboxylase Negative, argininedihydrolase Negative. will grow to CST completed with NaCl."

F. *Listonella Anguillarum:* " same how *vibrio ordalii,* apart from arginine dihydrolase positive."

G. *Yersinia ruckeri:* "Gram negative rod, motile, cytochrome oxidase negative, indole negative, alkaline/acid in TSI, ferments O/F-glucose. Biliary esculin and salicin negative.Two types, I and II, can be separated by the FAT or sorbitol utilization test (negative for kind I)."

H. *Serratia liquefaciens:* "Gram negative rod, motile, cytochrome oxidase negative, O/F fermentative, acidic/acidic or alkaline/acidic in TSI, lysine decarboxylase positive, arginine dihydrolase Negative, bile aesculin and salicin positive."

I. *Renibacterium Salmoninarum:* "small Gram-positive diplobacilli that only develop on KDM-2Medium. To confirm with FAT Where from PCR."

J. *Flavobacterium columnare* (flavobacteria): "Gram-negative, very long, thin rods, slippery Motility, colony is dry, rhizoid, yellowish on cytophage agar, often seen as matted crowds to fish gills."

K. *Flavobacterium psychrophilum* (flavobacteria): "Gram-negative, thin rod of medium length, sliding motor skills. Cultures produce non-diffusing yellow pigmented colonies with fine propagation edges. Cells become pleomorphic in older cultures. Confirm with Congo Red inhibition and PCR."

L. *Flavobacterium* sp.: "gram negative motionless Pole, Yellow, Orange, Where from pink colonies to TSA, variable cytochrome oxidase, O/F negative. Examine the gills for clubbing and threadlike rods."

5.5 BACTERIAL FISH DISEASES: CAUSAL AGENTS AND PLATES

DISEASE / SIGN

Bacterial Gil Illness: Causal officers: "*Flexibacter* sp. *Flavobacterium* sp. And unclassified filamentous bacteria, other unidentified non- mobile phone, mobile phone rods."

External panels: "gill hyperplasia; Swelling, sometimes clubbing, merge from filament and lamellae; presence from big pay from Bacteria; Apathy; loss from Appetite."

Bacterial kidney Illness: "Causal Agent: *kidney bacteria salmoninum."*

External panels: "exophthalmos; stomach Swelling;sometimes blisters on the skin filled with light amber to cream coloured purulent Liquid."

Internal characters: "pale and swollen kidneys; abscess inside kidney, liver or spleen; may have ascites fluid in abdomen; colon bloated, liquid completed."

Bacterial Sepsis: Pathogen : "*Aeromonas* or *Pseudomonas* sp.

in particular Aeromonas *hydrophila* and *P. fluoresces*. **External panels:** bleeding at based from fins, the eyes andvent blade gills; exophthalmos."

Internal panels: "bloody Where from ascites liquid in body Cavity; bleeding from internal organs; soft kidney; missing and other organs Blade."

Cold The water illness (and other proteolytic the skin infections):

Cause: "*Flavobacterium psychrophilum* and othersthreadlike Bacteria."

External panels: "tail blackout, White Where from bluish areas Behinddorsal Where from fat fins; loss from epidermis to dorsal Where from back side; Erosion of the dermis on the stem expose Skeleton- muscular; loss from caudal Stalk; erosion fromjaw Where from Snout; gill bleeding and Anemia."

Internal characters: "usually not noticeable, but sometimes: enlarged spleen with innumerable thread rods; petechiae bleeding from fat fabrics."

Enteric Redmouth: Causal Agent: *Yersinia ruckeri* types 1 and 2.

Outer Characters: "bleeding or erosion around the mouth; exophthalmos; inflated Abdomen; blush lid and finBase; inflamed hemorrhagic vent."

Internal characters: "Inflammation and bleeding in most visceral Organs; edema in lost, liver and Kidney; liver to be allowed to to be blade and hemorrhagic; liquids to be allowed to accumulate in stomach Cavity, stomach and Colon; inflamed, hemorrhagic lower colon with bloody Diarrhea."

Furunculosis: causal Agent: *Aeromonas salmonicida*.

External panels: "the skin blow Where from cooks which to be allowed to fester; petechiae; erythema from the eyes based from fins and analvent. in the acute Case, bleeding from the gills to be allowed to to be seen."

Internal signs: "renal necrosis; petechiae in the mesenteries About pancreas Stuff; localized bleeding in colonand Liver; dark, hypertrophy lost."

Vibriosis: Causal officers: *Listonella anguillarum*, *vibrio ordalii* and *vibrio* sp.

Outer Characters: "bleeding in the eyes; erythema based from fins; petechiae in the skin and musculature; blackout fromback surface; Bloating and open penetration of the abdomenCavity; bleeding at the vent blade gills."

Internal panels: " petechiae and hemorrhagic areas in internalorgans and mesenteries; enlarged lost and Kidney; liquid inthe Colon."

air bladder Where from colon Mushroom: Causal officers: *Phoma* sp. *saprolegnia* sp.

External panels: "trapped abdomen sometimes inflatedForward; vent erythema and rectal

prolapse; occasionally exophthalmos; swollen abdomen with open penetration of stomach Cavity."

Internal panels: " filled with liquid air bladder with bleeding and mycelium Dimensions; hemorrhagic colon with mycelium Dimensions; visceral Adhesions with diffuse fungal mycelium; second phone bacterial Sepsis."

Enteric Redmouth: Causal Agent: *Yersinia ruckeri* types 1 and 2.

Outer Characters: "bleeding or erosion around the mouth; exophthalmos; inflated Abdomen; blush lid and fin Base; inflamed hemorrhagic vent."

Internal characters: "Inflammation and bleeding in most visceral Organs; edema in lost, liver and Kidney; liver to be allowed to to be blade and hemorrhagic; liquids to be allowed to accumulate in stomach Cavity, stomach and Colon; inflamed, hemorrhagic lower colon with bloody Diarrhea."

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Internal signs: "renal necrosis; petechiae in the mesenteries About pancreas Stuff; localized bleeding in colon and Liver; dark, hypertrophy lost."

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air bladder Where from colon Mushroom: Causal officers: *Phoma* sp. *saprolegnia* sp.

External panels: "trapped abdomen sometimes inflatedForward; vent erythema and rectal prolapse; occasionally exophthalmos; swollen abdomen with open penetration of stomach

Cavity."

Internal panels: "filled with liquid air bladder with bleeding and mycelium Dimensions; hemorrhagic colon with mycelium Dimensions; visceral Adhesions with diffuse fungal mycelium; second phone bacterial Sepsis."

5.6 MYCOBACTERIA

Mycobacteria are straight or slightly curved, immobile rods, 0.1–0.6 × 1.0–10 μm in size. They are considered Gram positive, although they cannot be easily stained using this method. However, acid and alcohol resistance when stained by the Ziehl-Neelsen (ZN) method is the main feature of these aerobic microorganisms, which can be conveniently divided into slow-growing groups or fast-growing groups (Chinabut 1999). Mycobacterium piscium, the name given to the organism associated with the first observed case of mycobacterial infection in fish (Bataillon & Terre 1897), is no longer a valid species. Mycobacteriosis is a serious and often fatal fish disease affecting a wide range of species worldwide, both in culture and in the wild. The disease, caused by several species of the genus Mycobacterium, has received significant attention in recent years due to the discovery of new species in aquatic hosts such as Mycob. shottsii, Mycob. Montefi Orense and Mycob. neoaurum and acknowledging the very high incidence of infection in many populations that are widespread as part of the aquarium fish industry worldwide. Infection rates from studies of these fish have shown that on average 30% of all imports to Italy are infected (Zanoni et al. 2008) and there is little reason to believe that the situation is not the same in other importing countries. Interest has been fueled by the increasing incidence of animal diseases in wild fisheries and the ability of a few species to infect humans.

5.6.1 Mycobacterium marinum

Insulation

This bacterium was first isolated from marine fish in the Philadelphia Aquarium by Aronson (1926). M. platypoecilus, isolated from Mexican platyfish by Baker and Hagen (1942), is synonymous with M. marinum.

Habitat

Unknown, but carrier fish or subclinical fish are responsible for most infections in aquariums.

Morphology

The morphology is not characteristic. The bacilli can be up to 10 μm long and bead or stripe in color.

Culture

M. marinum is a slow growing organism. It can be cultured on general bacteriological media such as Brain Heart Agar or any egg or glycerol based medium used for isolating mycobacteria. However, comparative studies have shown that Sauton's modified medium (Chen et al. 1997) is best for homogeneous culture. Photochromogenic yellow colonies may be visible after 7 or more days of incubation at 20-30°C, but it usually takes 2-3 weeks for distinct colonies to develop. No growth occurs in the primary culture at 37°C.

Epizootiology

Infections with M. marinum occur in both tropical marine and freshwater fish, but not in species from temperate waters (Reichenbach-Klinke 1972). The disease is thought to be transmitted from fish to fish by ingestion of infectious material, although transovarian passage is possible, at least in viviparous species. Infections in carrier populations or chronic low-level clinical infections in breeding populations can be significantly exacerbated by breeding stress.

The microorganism also causes "pool granuloma" in humans, but infections can also be acquired in tropical fish aquariums (Swift & Cohen 1962; Black et al. 1971). It also causes a hypersensitivity rash on the arms of aquarists who expose themselves to infected water.

Clinical Pathology

Affected fish may be cachexic, darker in color, and have a swollen abdomen. At autopsy, miliary tubercles can be found in virtually any organ, but particularly in the liver, spleen, and kidneys (Fig. 8.48). Histopathologic findings vary, but ZN-positive bacilli are regularly seen (Fig. 8.49). Several authors consider the disease to be less cellular than tuberculosis in higher animals and dispute the presence of the Langhans giant cells characteristic of the mammalian tubercle (Sutherland 1922; Nigrelli & Vogel 1963). This has not been the experience of other investigators who have shown that caseation, typical production of Langhans giant cells, and cell-mediated immunity all occur at some point in the histopathogenesis of the M. marinum lesion, whether in marine or aquarium fish.

5.7 NOCARDIACEAE

Nocardia are gram-positive, aerobic, immotile actinomycetes with a complete life cycle including germination from quiescent microcysts, simple and complex cleavage and branching. Some species of Nocardia are acid-fast.

Nocardia Asteroids

Insulation

Nocardiosis caused by N. asteroides was first described in tropical freshwater fish by Valdez and Conroy (1963) (Figure 8.50). The same condition was reported by Snieszko et al. described in young rainbow trout. (1964), in Brown Trout by Campbell and MacKelvie (1968), in Largemouth Bass by Chen and Tung (1990), and in Formosa Snakehead by Chen (1992).

Habitat

N. asteroides can be isolated from soil.

Morphology

The microscopic appearance of these highly pleomorphic bacteria varies from small coccoid shapes to oval shapes and long, slender, branched, multiply septated filaments (Figure 8.45). They are generally acid fast when stained in tissues, but cultures may not show this property.

Culture

N. asteroides is an obligate aerobe that can be isolated on general bacteriological media. Irregular yellow-orange striped and ruffled colonies develop within 21 days after incubation at 18 °C. Aerial mycelium is usually produced along the colony edges.

CHAPTER 6

MUSHROOMS

Non-vascular plants, which differed from algae primarily in their lack of chlorophyll, were formerly defined by the term fungi. The absence of chloroplasts means that they cannot use photosynthetic pathways for energy and thus must inevitably lead a saprophytic or parasitic existence. However, with the advent of molecular taxonomic techniques, it has now been recognized that using a single collective term for such a widely separated group is inappropriate. Therefore, they are now commonly referred to as two distinct groups: fungi and oomycetes. There are at least 100,000 species of fungi and oomycetes, they show great morphological diversity, and some are responsible for a number of serious and economically important diseases of bony fish. Fish parasitic fungi are relatively few but notoriously difficult to classify, in fact many are classified as Fungi imperfecti (Deuteromycotina) as they are only known by their asexual (anamorphic) state, although they are believed to have a sexual (telemorphic) stage. Fungi and oomycetes that regularly parasitize fish are listed in Table 9.1. Diagnostically, they are often conveniently divided into two groups: those with transverse cell walls, representing the "septated" fungi, and those without, representing the "aseptated" species, members of which can be found among the two. fungi and oomycetes.

6.1 OOMYCETES

Oomycetes are one of the most important groups of fish pathogens (Neish & Hughes 1980). Although they look like fungi, they are actually closer to golden algae and belong to the chromista or chromoalveoli and are therefore not "true fungi". They are classified as Stramenopiles (heteroconts), which also includes golden brown algae and diatoms (Baldauf et al. 2000). Taxonomically, Oomycetes are divided into three subclasses: Saprolegniomycetidae, Hipidiomycetidae and Peronosporomycetidae. Most fish and animal pathogens Oomycetes belong to the Saprolegniomycetidae, which has two orders: Saprolegniales and Leptovitales. Three main genera, Saprolegnia, Achlya and Aphanomyces, are recognized within the Saprolegniales. All are capable of infecting fish or shellfish (Daugherty et al. 1998). Some Saprolegnia species, including S. ferax, are believed to be partially responsible for amphibian declines in natural ecosystems (Pounds 2001; Kiesecker et al. 2001). The related species Aphanomyces invadans drastically altered freshwater teleost populations in South and Southeast Asia in the 1980s (Roberts et al. 1986), and Aphanomyces astaci, which caused the

destruction of crayfish populations in natural settings, is one of eight microbial species on the international list of the world's 100 most unwanted extraterrestrial invaders. The clinical picture induced by oomycetes is usually characteristic and according to Ainsworth (1976) the oomycete infection of the cockroach illustrated by Arderon in 1748 represented the first clinical manifestation of oomycete infection in vertebrates. Oomycetes are widespread in aquatic habitats and very few are parasites. However, all share the common property of producing biflagellate motile spores. Asexual reproduction by zoospores produced in a zoosporangium is the primary means of dispersal, but sexual reproduction by the fusion of two gametes into a thick-walled oospore, or quiescent spore, is the reason for the class name Oomycetes. The filaments of oomycetes, called hyphae, are aseptic (that is, they have no transverse walls).

Table 9.1 Oomycetes and fungi regularly parasitizing on fish

Oomycetes	Saprolegniales	*Saprolegnia* *Achlya* *Aphanomyces* *Branchiomyces*
Chytridiomycetes	Chytridiales	*Dermocystidium*
Zygomycetes	Entomophthorales	*Ichthyophonus* *Basidiobolus*
Deuteromycetes (= *Fungi imperfecti*)	Moniliales	*Exophiala* *Aspergillus*
	Sphaeropsidales	*Phoma*

6.2 SAPROLEGNIALS AND SAPROLEGNIACEAE

Although there are four orders within the Oomycetes, almost all important fish pathogens belong to the family Saprolegniaceae, the most important being the genera Saprolegnia, Achlya and Aphanomyces. The taxonomy of members of the Order is complex, but is gradually becoming clearer through the use of molecular methods of kinship attribution.

Oomycetes are fungus-like protists rather than true fungi and are therefore classified alongside diatoms, brown algae, and golden algae. In the past, they were often referred to as pseudomycetes (Cavalier-Smith 1987). Admittedly, the Saprolegniaceae are ‘aquatic molds’ that have a profusely branched, unchambered mycelium that looks like tufts of cotton wool in water.

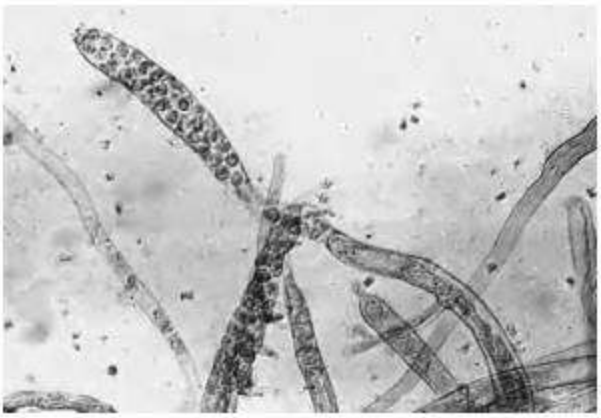
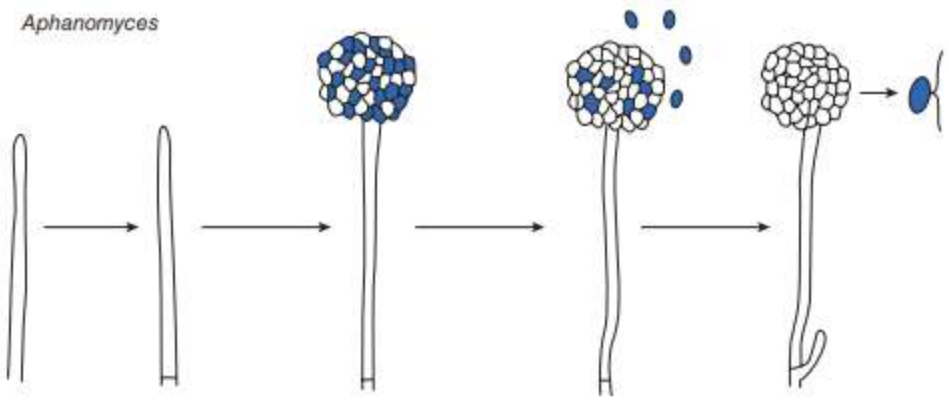


Figure 9.1 Wet preparation of Saprolegnia spp. a skin lesion of an Atlantic salmon showing unseptate hyphae and an asexual sporangium containing motile zoospores.

The shape of the hyphae varies considerably from species to species, but all contain cellulose. Although the hyphae are not septate, the reproductive structures are separated from the somatic hyphae by a septate zoosporangium containing biwinged zoospores (Figure 9.1). The pattern of sporangia dehiscence and zoospore behavior distinguish different species, but in Saprolegnia and Achlya it is usually also necessary to preserve the oogonium in order to distinguish them by sex structure (Figure 9.2). Some species are found in brackish water, but salinity levels above 2.8% limit their distribution



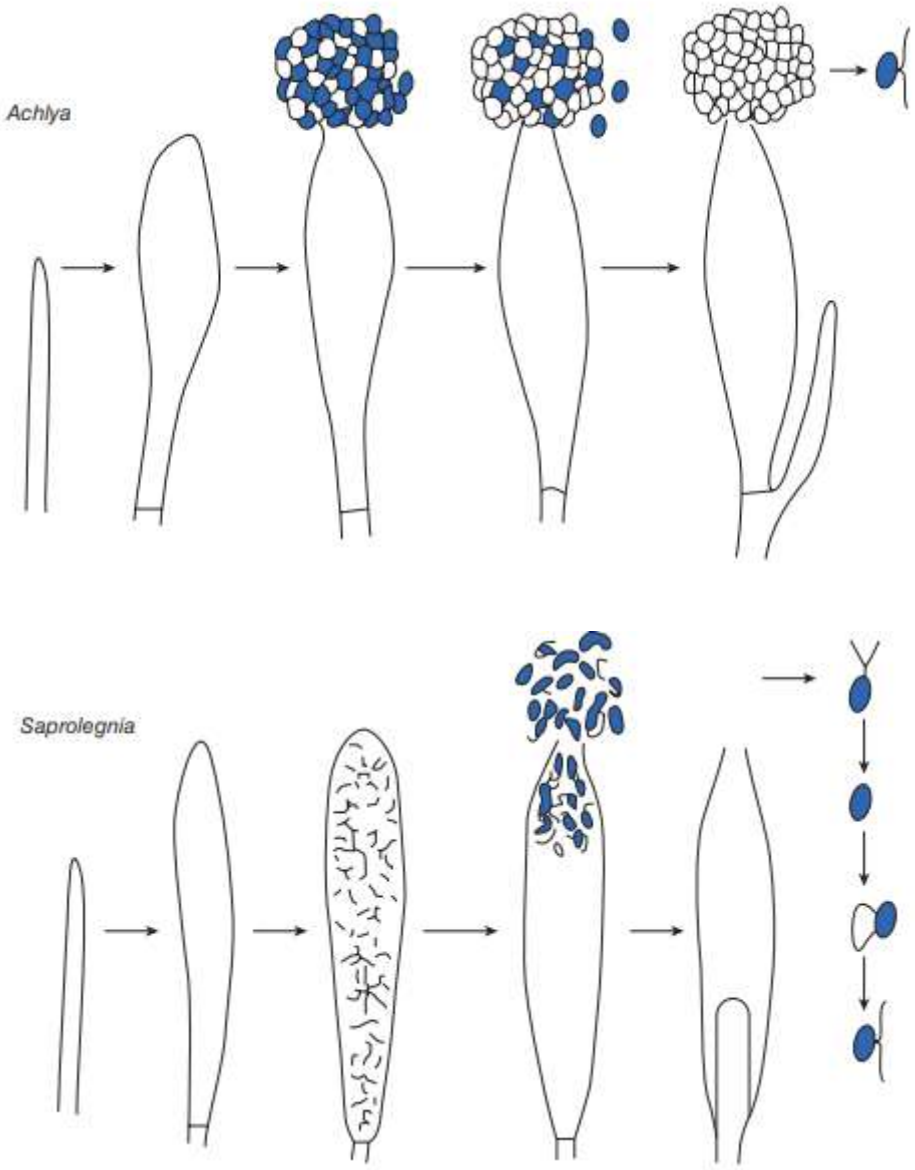


Figure 9.2 Formation and dehiscence of zoosporangia in Aphanomyces, Achlya and Saprolegnia. (Courtesy of Dr. LG Willoughby.)

More details on their classification are

6.2.1 Saprolegnia spp.

Although this clade is one of the most important in fish pathology, its taxonomy has long been the most confusing. The reasons for this are varied, but historically the confusion began in the late 19th century, when considerable interest arose in an epizootic Atlantic salmon associated with an oomycete invasion (Huxley 1882; Murray 1885). At the time, the oomycete identified as Saprolegnia ferax was believed to be solely responsible for the disease, but it is believed

these researchers are now studying the later stages of the disease, now known as ulcerative dermal necrosis (UDN).

The species was later defined as Saprolegnia parasitica by Coker (1923) to account for all asexual isolates of Saprolegnia that parasitized fish. Subsequently, the general consensus, using Oogonium-based morphological criteria, was that the taxon S. parasitica should be reduced to a synonymy with the common saprobiotic species Saprolegnia diclina, sometimes referred to as the Saprolegnia parasitica-Saprolegnia diclina complex.

Therefore, the exact taxonomy of the fish-pathogenic saprolegnia has been very confusing for many years, so much so that Di é guez-Uribeondo et al. (2007) noted with some justification that identifying parasitic Saprolegnia isolates at the species level using traditional taxonomic criteria and keys was at best problematic and at worst impossible.

To clear up this confusion, Diéguez-Uribeondo and colleagues (2007) performed a detailed study identifying both phylogenetic and taxonomic aspects within the S. diclina-S. parasitica species complex were combined. They sequenced the internal transcribed spacer of nuclear ribosomal DNA and examined cyst ornamentation, reclusive germination and zoospore reappearance ability in a large and representative sample of isolates of Saprolegnia spp. and the S. diclina-S. parasitica complex originating from diverse hosts and geographic origins.

Their results supported an earlier suggestion by Beakes et al. (1994) to assign the names S. parasitica to parasitic isolates recovered from lesions on live salmonids and other fish with characteristic tufts of hair and a withdrawn germination pattern. They also agreed that Saprolegnia parasitica should be recognized as a separate taxon from S. diclina.

6.2.2 Saprolegniosis

This term describes an infection with Saprolegnia parasitica. It usually leads to surface infection, but the pathogenesis is complex and the precise role of the oomycete is regularly debated. Important for the pathogenesis, however, is the effect of the prominent reflexed hairline roots on the surface of secondary cysts, which can act as a ridge (Fig. 9.3) (Pickering & Willoughby 1982). Another element could be the recently described effector protein, which is produced by hyphae during a biotrophic stage of the infection process. This is similar to biotrophic and hemibiotrophic plant-pathogenic oomycetes and suggests that the pathogen may

have an early stage of infection during which it does not kill host cells but keeps them under control.

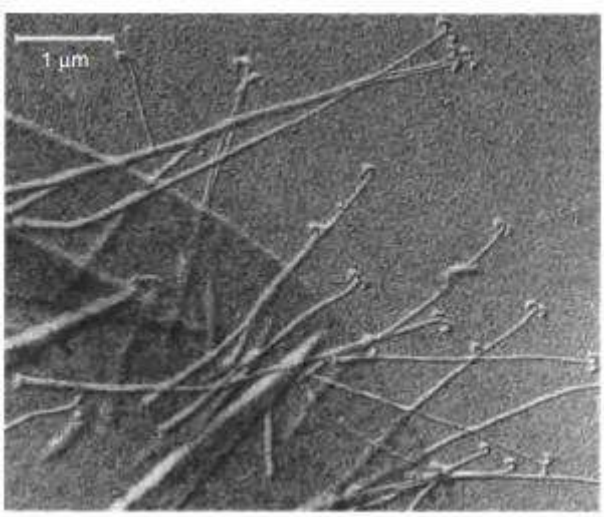


Figure 9.3 Hook hairs in a saprolegnia cyst.

1. Predisposing Factors. Several factors are involved in the development of oomycete infection in fish. These factors can act on the fish or on the pathogen - and it is a combination of factors rather than a single condition that ultimately leads to infection. The pathogens responsible for saprolegniosis have long been thought to be secondary pathogens, and lesions are commonly observed after handling and after any traumatic skin injury as a result of hormonal changes in the skin associated with smolt or egg laying and associated with pollution or bacterial or viral diseases.

   Primary saprolegniosis was described by Hoshina et al. (1960) and Hoshina and Ookubo (1956) in cultured eels with no visible anterior fish injury. Similarly, Tiffney (1939) demonstrated Saprolegnia invasion in a variety of fish with no apparent prior injury. However, microscopic lesions may have been present, and Tiffney found with certainty that macroscopic lesions greatly increased the likelihood of infection.

   Work by Richards and Pickering (1978) showed that outbreaks of saprolegniasis in spawning brown trout almost always involved some form of saprolegnia with a low degree of homothallic sexuality. This oomycete, despite prolonged incubation on various media, appears unable to produce sexual structures except to a limited extent at low temperatures. This supports the results of Willoughby (1969) who systematically isolated a similar sterile form of Saprolegnia from UDN lesions in Atlantic salmon. The

infective stage of the oomycete is the zoospore, as conclusively demonstrated by Nolard-Tintigner (1973) in experiments with a variety of oomycetes to infect guppies and swordtails.

Temperature has a significant impact on the development of Saprolegnia infection. While infection following trauma can occur at any temperature compatible with fish life, most animal diseases occur when temperatures are low for this fish species. Hoshina and Ookubu (1956) and Hoshina et al. (1960) pointed out that sure, a saprolegniasis of eels, stopped when the water temperature exceeded 18°C. However, high temperature stress can also induce Saprolegnia invasion. Roth (1972) noted that infection of experimental white suckers usually occurred when the temperature exceeded 10 °C.

In the southern United States, channel catfish are commonly afflicted with a saprolegniasis known as winter kill, resulting in large economic losses. Affected fish show patches of Saprolegnia on the skin in winter and also develop endophthalmitis (MacMillan 1985). A specific species is believed to be involved, although their taxonomy has not been fully defined and the low temperature suppresses the normal structural and immune mechanisms that would control them.

Although the skin cuticle itself is thought to possess some anti-oomycete activity (Willoughby 1969), spawning salmonids have a particularly well-developed cuticle, and yet infection with Saprolegnia is common. Similarly, precocious salmonids often develop infections, unlike immature fish kept under identical conditions. The increased thickness and mucus production of the skin of sexually mature salmonids are sex hormone-mediated effects, and steroid metabolic changes have been shown to occur directly in the epidermis of salmonids in relation to sexual maturity (Hay et al. 1976). Experimentally, injection of various hormones facilitated the induction of oomycete infections in fish.

2. Clinical Features. Saprolegnia lesions are focal gray and white patches on the fish's skin that have a cottony appearance when examined underwater, where the hyphal filaments extend into the water. Early lesions are often nearly circular and grow around the periphery by radial expansion until the lesions coalesce (Fig. 9.4). At this later stage, oomycete plaques are often dark gray or brown in color as the mycelium traps mud or

silt. Although the distribution is mostly random, certain parts of the body can be particularly affected, for example the head region in secondary UDN infections of salmonids and safe eels. Skin and gill lesions are by far the most commonly observed, but cases of internal organ infections have also been reported.

Figure 9.4 Spawning brown trout with Saprolegnia growths on the head and back. The latter in particular shows the lively colony growth characteristic of this pathogen.

Gill infections in juvenile salmonids often originate in the mouth or gill cavity and can cause high mortality in juvenile fish reared in surface water hatcheries. Agersborg (1933) reported an intestinal infection in small brown trout by Saprolegnia ferax [sic] and a similar infection by Aphanomyces spp. was reported by Shanor and Saslow (1944). GD Cawley (personal communication) and Roberts (unpublished) also observed peritoneal saprolegniosis in salmon and trout fry, which is usually seen when the juveniles still have remnants of the yolk sac. Infection usually occurs via the buttocks into the epidermis or, in the latter cases, via the intestines. There are no reports of infection of internal organs via the vascular route, although Nolard-Tintigner (1973) described invasion of blood vessels and consequent thrombosis, a condition more usually associated with infection by Branchiomyces spp. Saprolegnia is also a common invader of incubating fish eggs. It generally settles on dead eggs first and then spreads to adjacent healthy eggs. The time scale for lesion development varies considerably with environmental conditions. Infection by salmonids can sometimes be fatal within 36 hours of initial infection, particularly if the gills are affected.

3. histopathology. The oomycete pathogen typically establishes focally, invades the spongy layer of the dermis, then spreads laterally across the epidermis, eroding it as it spreads. Relatively superficial invasion of the dermis quickly leads to fluid imbalance

and peripheral circulatory failure (shock) due to the inability to maintain circulating blood volume.

In more chronic cases, usually when the accompanying environmental stresses are not as severe, the mycelium can penetrate the dermis and migrate between the interyotomal fascial planes. Bacterial infection can occur with such chronic lesions.

Although generalized systemic infections are less commonly reported, Nolard-Tintigner (1973) described necrotic lesions of the spinal cord associated with nervous signs and thrombotic mycelial occlusions of blood vessels in experimental studies in guppies. However, they are very small fish with relatively short distances between skin and spinal cord.

In hematoxylin and eosin stained skin sections infected with Saprolegnia, numerous hyphae are observed on the skin surface entangling cellular debris and water-entrapped materials by the hyphal strands (Figure 9.6). Beneath this superficial carpet of mycelium are areas of degenerating tissue ranging from superficial skin necrosis and edema to deep myofibrillar necrosis and extensive hemorrhage. However, the majority of lesions are superficial, and often the only effect seen at the dermal level is a swelling artifact, which is evident by variations in staining affinity, with collagen fibers becoming more basophilic. Often only a mild inflammatory response is present, but when concomitant bacterial infection occurs, particularly at elevated temperatures, a distinct inflammatory infiltrate is usually visible. Oomycete hyphae are PAS positive and are easily detected by silver impregnation methods such as the Grocott technique. They are branched, non-septate and about 20 μm in diameter.

4. Insulation. A variety of methods can be used to obtain bacteria-free Saprolegnia colonies, all of which involve the use of "baits" followed by agar culture.

5. prophylaxis and treatment. Disease prevention can be aided by keeping the fish in good housing conditions. Proper feeding, crowd avoidance, and good water quality are essential, but even then, mature male salmonids can still succumb. When fish develop saprolegniosis, a variety of external disinfection treatments can be used. These include malachite green, copper sulfate, potassium permanganate, salt and formalin. Malachite green has long been the treatment of choice (Willoughby & Roberts 1992), but its potentially mutagenic or teratogenic effects have made its use illegal worldwide.

6.2.3 Aclya spp.

The genus Achlya includes a number of fish-parasitic species (Neish & Hughes 1980). In contrast to Saprolegnia, the zoospores of Achlya do not move away from the zoosporangium, but enclose themselves as a hollow sphere at its mouth. Thus there is no freely motile primary zoospore; infective secondary zoospores emerging from the cyst arise directly from the mouth of the zoosporangium.

Although there have been numerous reports of infection of fish by various Achlya species, there is no consistent and regularly observed clinical condition as is the case with Saprolegnia. However, Nolard-Tintigner (1973) suggested that Achlya and Dictyuchus sp. were more important than Saprolegnia as a cause of death, at least in the tropics. This was supported by the work of Srivastava and Srivastava (1978) who showed that Achlya is highly pathogenic for traumatized Puntius sp. And Colisa sp.

Aphanomyces spp. Only one, truly clonal, species of Aphanomyces, namely Aphanomyces invadans (Willoughby et al. 1995), was responsible as a necessary cause, aided by a variety of secondary pathogens including other oomycetes, fungi, bacteria and parasites, most importantly the modern fish pandemic. Epizootic ulcerative syndrome (EUS), a disease of estuarine and freshwater fish characterized histologically by prominent mycotic granulomas, has spread since 1971 from Queensland, Australia and Japan to Papua New Guinea, the Philippines, Indonesia, Malaysia, Thailand, Burma and the Native Americans spread to the subcontinent (Roberts et al. 1986). It is now also common in the Mediterranean and Africa, and has spread from the Chesapeake Bay region of the United States across the east coast to the pond fish production areas of the southern United States (Blazer et al. 1999; Vandersea et al. 2006).

Economic losses have been estimated at at least US$10 million per year since the 1980s. The disease continues to spread west, and as it affects a wide range of widely exported aquarium fish, among others, it seems likely that its reach will continue stretch. Almost all species of freshwater and brackish water fish can be infected, but some species such as mugil cephalus, snakeheads and Indian carp, all important food species, are particularly susceptible (Roberts et al. 1994).

Aphanomyces invadans has been extensively characterized (Lilley & Roberts 1997; Calinan et al. 1995) and genetic analysis has shown that all strains are identical regardless of fish species

or geographical area of origin (Lilley et al. 1997). It differs from other tropical Aphanomyces species by its slow growth and delicate hyphae and, above all, by its acute pathogenicity under experimental conditions, which reflects the clinical situation.

Epizootic ulcerative syndrome, when first appearing in a new area, is characterized by a wave of acute death in large numbers of wild and farmed fish, with large, flat, gray or red ulcers, often with a brown necrotic center. Typically, ulcers are found on the side of the body, and in one particular type, all affected individuals can have lesions in the same location. Some species die very quickly, but a few, particularly snakeheads (Channa spp.), take much longer to die and can display a wide range of extensive and often grotesque clinical symptoms (Figures 9.7a, 9.7b, 9.7c, and 9.7d). These include complete posterior body erosion and necrotic destruction of the skull bones to expose the surface of the brain.

The predominant clinical and histopathologic feature in all cases is the extensive ulcerative lesion superimposed on a penetrating myopathy extending deep into the muscle. It has an off-white necrotic superficial covering of degenerating tissue and fungal hyphae. Below is an area of occulent Myofi Brilliant necrosis with little cellular response. However, once the lesion has matured, the oomycete penetrates deep into the tissue and may invade the spinal cord or abdominal viscera. It is normally lined with a thick layer of chronic inflammatory cell tissue composed primarily of host macrophages and epitheloid cells.

The resulting widespread fungal damage is ultimately enough to kill most fish, but other opportunistic oomycetes, fungi, bacteria (particularly Aeromonas hydrophila), and protozoa also usually contribute to the ultimate fish kill.

6.3 BRANCHIOMYCES SPP.

It is generally believed that there are two species of Branchiomyces, but both are known only as parasites of the gill tissue of fish. Both have branched coenocyte hyphae that produce aplanospores by endogenous cleavage. They are usually separated according to the size of the spore wall and hyphae, and specific habitat in the gills. However, it is possible that both variants are of the same species and that growth type and morphology are in part a function of growth location and not an associated taxonomic trait.

Both species – Branchiomyces sanguinis, which is found in the blood vessels of the gills, and B. demigrans, which can penetrate the gill tissue to the surface - are both commonly associated

with infection of carp fish (Schaperclaus 1954; Reichenbach-Klinke 1973). They are also recognized in the Indian subcontinent (Hara & Pillay 1962) and Japan (Egusa & Ohiwa 1972). The branched, unseptate hyphae of B. sanguinis are 8–30 μm in diameter and spores are 5–9 μm in diameter. Those of B. demigrans are larger.

Branchiomyces spp. are relatively easy to grow on agar medium (Peduzzi 1973), but no critical taxonomic analysis has been performed. Peduzzi is the only researcher to have systematically studied the organism, and his antigenic studies in particular have suggested that members of the clade are oomycetes within the Saprolegniales.

Branchiomycosis Also known as gill rot, this disease is characterized by infarct necrosis of the gills due to intravascular growth of Branchiomyces spp. Both species of Branchiomyces can be involved in the disease.

The first record of branchiomycosis is by Plehn in 1912. Common carp are most commonly affected, but the disease has also been observed in tench and stickleback, Japanese eel and Indian carp. In tench, B. sanguinis and B. demigrans have been found to infest the same fish. The latter organism was first described by Wundsch in 1930 and causes gill rot in northern pike.

Histologically, hyperplasia, gill lamellae fusion, and massive necrosis due to vascular thrombosis caused by fungal hyphae are associated with telangiectasia and vascular necrosis. B. sanguinis does not grow well outside of blood vessels, in contrast to B. demigrans, which grows through the vessel wall as a mass of hyphae and penetrates necrotic tissue. Affected fish can die as early as 2 days after infection and there can be up to 50% morbidity.

Infection probably occurs through spores released from necrotic gill tissue, but it is unclear whether infection occurs directly through the gills or hematogenously after ingestion of the spores. Plehn (1912) suspects that the reason for the localization in gill vessels is that B. sanguinis can only grow in areas with high oxygen tension. B. demigrans probably has a lower oxygen requirement. Schaperclaus (1954) suggested that the disease is favored by water rich in organic fertiliser, algal blooms and temperatures above 20 °C. Grimaldiet al. (1973) went further and related wild fish outbreaks in southern European lakes to eutrophication.

There is no proposed treatment for the disease. Prevention can only be achieved through strict sanitation, removal of dead fish and avoidance of overfeeding, especially in high water

temperatures. Increasing the water supply often proves beneficial during attacks, and eradication of the disease can be attempted by draining and liming affected ponds.

6.4 CHYTRIDIOMYCETES (TRUE FUNGI)

6.4.1 Chytridia

dermocystide

This genus was established by Pérez in 1907 and the life cycle of the type species was described by him in 1913. Since then, several isolates of a number of species of Dermocystidium have been made from a variety of fish (reviewed by Pauley 1967). There has been much debate as to whether the organism is in fact a fungus or a haplosporidian (Lom & Dykova 1992). Reichenbach-Klinke and Elkan (1965) assign the organism to haplosporidia, while Pauley (1967) and Allen et al. (1968) classify the organism as a fungus. These organisms are closely related to Dermocystidium marinum, a causative agent of oysters responsible for considerable losses and classified as a marine phycomycete by Johnson and Sparrow in 1961.

Pauley (1967) described an outbreak of Dermocystidium infection in adult Chinook salmon causing 25% mortality in 5000 fish. Alle et al. (1968) described a similar outbreak in adult Chinook salmon and fry, and also reported disease in coho and sockeye salmon. Outbreaks appeared to be most severe at temperatures below 15°C.

In adults, numerous small cysts, about 1 mm in diameter, occur in the gills. These resemble lesions of Epitheliocystis and contain large numbers of protozoa 5–8 μm in diameter (hypnospores). These have an eccentric core, a large vacuole and an inclusion body (volutin body). The cysts have a thin capsule of fibrous tissue and produce a marked inflammatory response and hydropic degeneration and hyperplasia of the branchial lamellar epithelium. There are also lesions of the spleen with congestion and fibrosis around the colonies of Dermocystidium. The parasites exert an even more drastic effect on the young fish. Severe gill infestation can often physically prevent closure of the operculum, and extensive fin and skin involvement often occurs. Death usually results from anoxia. In Atlantic salmon parr, it causes mortality associated with visceral caseation of infected nodules.

The organism can be grown on a variety of media such as thioglycolate agar (Ray 1952) with the addition of antibiotics to prevent overgrowth of contaminating bacteria.

6.5 ZYGOMYCETES (TRUE FUNGI)

6.5.1 Entomophthorales

Ichthyophonus hoferi

This organism was originally assigned to the Haplosporidia and named Ichthyosporidium gasterophilum when it was described by Caullery and Mesnil (1905). Laveran and Pettit (1910) and Plehn and Mulsow (1911) recognized a similar organism as a fungus and named it Ichthyophonus hoferi after Hofer, who first described it as a haplosporidia affecting plaice, trout, sea bass and whiting. Since those early days, the two names have been used interchangeably, and while consensus favors the classification of the organism as a Phycomycete of the Entomophthorales, there is still considerable debate as to its precise taxonomic classification. Sprague and Vernick (1974) describe the electron microscopic appearance of Ichthyosporidium and assign it to the microsporidia.

The current position seems to be that there are many similar microorganisms that are probably related, almost certainly fungal in nature, and associated with similar diseases in different species of fish. The life cycle is believed to vary between "strains" (or species?) and between species of fish. McVicar (1999) reviewed the spectrum of infections and detailed the life cycle as it occurs in marine fish.

There are a number of stages in the parasite's life cycle and its microscopic appearance varies depending on the stage present in the tissues and in different hosts.

The "dormant" or spore stage is most commonly observed. This is generally spherical or oval with a diameter of 10 to 250 μm. It has a double wall that colors PAS positive and also silver color positive. According to Wolke (1975), the spore cytoplasm is often vacuolated, weakly basophilic, PAS-positive and argyrophilic, and multinucleated. These spores can be present singly or in large numbers in different organs and are often associated with other stages (Fig. 9.9).

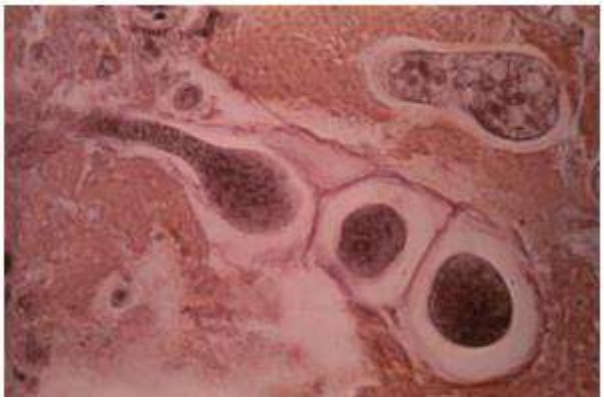


Figure 9.9 Spores of Ichthyophonus hoferi in herring tissue. H+E×100.

The germinating spore can be seen in cross section and is often observed post mortem. It consists of a cytoplasmic extension bounded by the inner spore wall, which breaks through the thicker outer wall (Fig. 9.10). This is followed by further differentiation into non-septated macrohyphae of up to 40 µm in width. New spores, called hyphal bodies, can also form from the hyphae.

According to Dorier and Degrange (1961), salmonids are first infected orally. The latent cysts of the digestive tract can then form amiboblasts, which, after rupture of their outer wall, give rise to amoeboid embryos. These penetrate the mucous membrane and enter the bloodstream to be spread to other organs, particularly the muscle, forming new cysts which then further parasitize the surrounding tissues by forming plasmodia or endospores. Alternatively, latent cysts in the intestine can germinate and invade the intestinal wall itself. They also reach the surface and, when patenting, release infectious spores into the external environment.

The organism can be grown on fungal media such as Hagem medium or Sabouraud dextrose agar slants supplemented with 1% bovine serum (Sindermann & Scattergood 1954) or in tissue culture medium (McVicar 1982). Growth is plentiful in 7 to 10 days at an optimal temperature of 10°C.

6.6 ICHTYOPHONOSIS

The disease caused by Ichthyophonus is a systemic granulomatosis and is found in many species of freshwater and marine fish. It has been the cause of the death of large numbers of Atlantic herring in frequent disease outbreaks along the east coast of the United States. Wolke (1975) mentions such epizootics where about 25% of the fish were infected compared to less than 1% morbidity in years without epizootics. Herring outbreaks typically occur in winter and

spring, and infection manifests as a rough skin texture, described as “sandpaper effect”, occurring mainly in the lateral-ventral area of the tail (Hodneland et al. 1997). It is believed that macroscopic lesions in the skin appear as early as 30 days after experimental feeding of the fungus.

The sandpaper effect is caused by epithelial loss in proliferating dermal fungal granulomas. These are usually black, about 1mm in diameter, and rise above the surface of the skin. Further growth of the fungus causes local necrosis and leads to the formation of abscesses or ulcers. In other species of fish, the internal organs are affected more often than the skin, and this internal infection manifests itself as raised white nodules that closely resemble tuberculosis granulomas. They are found in all organs, especially the heart and liver. Infections in freshwater rainbow trout.

The heart, liver, muscles, kidneys, spleen and even the brain can become infected, and signs obviously vary depending on the extent of damage and the organ or organs affected. The host's response to the parasite varies, but a severe granulomatous reaction is the usual finding, with large numbers of epitheloid cells and macrophages and occasionally giant cells. In the early stages, cells of the inflammatory series are observed in large numbers. Granulomas usually have a well-developed connective tissue capsule and sometimes the spores are surrounded only by a capsule of fibrous tissue. Kocan et al. (2006) showed that a major effect of infection in wild salmonids is a severe reduction in cardiac output and hence swimming ability in infected fish.

Initial infection likely occurs in at least three different ways. Infected material can be swallowed, infected fish can be swallowed (this has occasionally happened when infected "garbage fish" have been fed to intensively farmed salmonids), or infected copepods can be swallowed. Reichenbach-Klinke and Elkan (1965) discuss several cases of isolation of the fungus from copepods, although experimental feeding of plankton exposed to Ichthyophonus to herring did not result in infection. Experimental feeding of infected fish material regularly leads to infections and McVicar (1982) has extensively described the pathogenesis and pathology of these infections in trout and flatfish.

Pumpkin preparations made from fresh material show the spores and often germinating spores are visible. These are considered diagnostic. The onset of germination is believed to be due to the increase in CO 2 levels in the tissues as they degenerate.

Measures to prevent infection with this pathogen, including steam sterilization of waste fish, are essential as treatment is impractical. A method of treating aquarium fish using phenoxethol is cited in Reichenbach-Klinke and Elkan (1965), but it is believed to be effective only in the early stages of the disease. Elimination of spores that are in the infection stage can be eliminated by treatment with sodium hypochlorite and polyvinylpyrrolidone - iodine.

6.6.1 Basidiobol

The status of this genus as a pathogenic fish is uncertain. Like Ichthyophonus, a member of Zygomycotina: Entomophthorales, it is a group known for its pathogenicity to insects. Basidiobolus ranarum, a fungus regularly isolated from frog dung, has been associated with fish (Nickerson & Hutchison 1971) and B. meristophorus with young carp and their eggs. However, Neish and Hughes (1980) doubt whether it makes sense to consider any basidiobolus species as a true fish pathogen.

6.7 DEUTEROMYCOTINA (imperfect fungi)

Fungi imperfecti are fungi united by the common trait of having no sex stage (telomorph). Many fish infections caused by these fungi have been described. Although generally considered to be opportunistic pathogens, such infections are usually chronic, progressive, and fatal. When widespread infections occur in farmed fish, the losses can be economically catastrophic. Because there is no formal taxonomic scheme for the various pathogens or infections associated with this idiosyncratic grouping, they are described here by clinical status. All are associated with systemic granulomas.

Diseases such as aspergillomycosis (Olufemi et al. 1983) and fusarium described by Horter (1960) which affect carp may also be included under this heading. Many "tumors" have also been linked to fungi. They are almost always chronic fungal granulomas. However, in most cases only fixed material was available for investigation, so that isolation, typing or pathogenicity studies could not be carried out on the fungus in question.

6.8 ASPERGILLOMYCOSIS

Many members of the Moniliaceae can be pathogenic and because of the problems encountered in finding sex stages in many of them, the group is usually assigned to Deuteromycotina or Fungi imperfecti. However, many imperfect "stems" (or species) show

very close similarities in their anamorphic structures to known examples for which telomorphic structures have been described, and should be ascribed to Ascomycotina, terrestrial or aquatic septate fungi whose sex involves Ascomycotina producing an ascus with ascospores. The name Aspergillus derives from the distinctive nature of the spore stalks and heads, which resemble the bottle brush or holy water brush with which the priest Micheli (1729), who gave the name, would be familiar.

Aspergilli are ubiquitous and mainly involved in saprophytic degradation processes. A number of species have been implicated in diseases of the pulmonary system in higher animals (Austwick 1965) and of course the by-products of Aspergillus degradation of fish feed, aflatoxins, are responsible for aflatoxicosis in fish. However, their role as pathogens in farmed fish was not recognized until 1983 by Olufemi et al.

Raper and Fennell (1965) identified 132 species and 18 varieties of Aspergillus characterized by culture color, morphology and size of the conidia and various parts of the conidiophore (e.g. phialides and vesicles). They can be easily isolated from infectious lesions by concentrating and culturing on Sabouraud or Czapek medium at 30°C in the dark (Olufemi & Roberts 1983).

Aspergillomycosis has so far only been described in cultivated tilapia (Olufemi 1985). In natural outbreaks on farms, the condition manifests as a sudden increase in mortality after any husbandry stress. This is associated with bloating, darkening of color and lethargy. Incision of the abdominal cavity results in the release of copious amounts of fluid, and on autopsy the liver shows severe focal necrosis. Mortality can be 20% of the stand or more, but losses are usually sporadic throughout the growing season.

Histopathologic features show the presence of an obvious fungus with septate hyphae, but usually no other pathogen. The liver, spleen, kidneys and intestines also contain fungi, as does the swim bladder. Initially, the hyphae appear to spread unhindered (Fig. 9.11), but in later stages the organisms become entrapped in chronic inflammatory granulomas (Fig. 9.12) (Olufemi & Roberts 1986). Infection occurs through contaminated food and several species may be involved, but Aspergillus flavus and A. niger are the most commonly observed.

6.9 SYSTEMIC FISH FISH MYCOSIS

In 1969, Fijan described a condition in channel catfish in which skin ulcers 2–15 mm in diameter and up to 5 mm deep were found associated with adhesions and peritonitis, suggesting

both hematogenous spread and local extension. Tubular, branched, septate, PAS-positive hyphae were found in the nodules and identified as belonging to the Dematiaceae family. The disease was reproduced in channel catfish, white channel catfish and sunfish by intraperitoneal injection of fungal material and the organism was reisolated and identified.

Necrotic foci containing hyphae and a mixture of cellular and caseous material were found in experimental fish. These lesions had thick walls and giant cells were present in the wall. No central nervous system effects were observed in this outbreak.

6.10 CEREBRAL MYKETOME

Carmichael (1966) described a Phialophora-like fungus causing epizootic so-called cerebral mycetoma in cutthroat trout. The organism was named Exophiala Salmonis, the lesion being a chronic, nonsuppurative granuloma with the presence of numerous giant cells in the brain and cranial region. Langdon and McDonald (1987) described Exophiala pisciphila infection causing high mortality in 1+ Atlantic salmon. The hyphae invaded the head, lateral line, and semicircular canals and elicited a widespread granulomatous inflammatory response with cartilage necrosis.

6. **11 SYSTEMIC PHIALOPHORA INFECTION**

Ellis et al. (1983b) provided one of the most detailed descriptions of systemic mycosis in their study of Phialophora infection of Atlantic salmon parr. Infection mainly occurred in January when the water temperature was low. Affected fish presented petechiae on the fin bases and ventral surface with pink inflammatory edema of the abdominal cavity, swelling of the kidneys and characteristically deflation of the swim bladder. Whitish masses consisting of dense clusters of mycelium were found on the surface of visceral organs, and hyphae and conidia were also found in the lumen of the collapsed swim bladder.

The fungus possessed a thin-walled, septate-branched mycelium, and based on this and the fact that the vast majority of conidiogenic phialides had ruffs, Ellis et al. (1983b) assigned it to the genus Phialophora, but recognized the unsatisfactory nature of the systematics of these fungi.

6.12 SCOLECOBASIDIUM HUMICOLA INFECTION

Ross and Yasutake (1973) described systemic mycosis in coho salmon kept for experimental purposes. The organism was previously described from the ground (Barron & Busch 1962) and has since been described in frogs.

An enlarged abdomen was usually observed in affected fish, often accompanied by skin lesions. Ascites, adhesions and gray areas in the internal organs, especially the kidneys, were also frequently observed.

Histopathologic features were similar to those described by Carmichael (1966) for E. Salmonis infection, but no cerebral mycetomas or giant cells were observed. In larger lesions, branching hyphae and lymphoid infiltration were observed along with areas of necrosis.

The disease could not be transmitted experimentally by including the fungus in the normal diet, but transmission was achieved when ground glass was also added to the diet. The morbidity of the disease in natural herds was low. When grown on Sabouraud's Dextrose Agar, the colonies were filamentous and olive in color with a powdery surface. Older colonies were olive black with brown aerial hyphae. Conidia were usually unsepate and appeared singly.

6.13 SPHEROPSIDAL INFECTION

Phoma herbarum, a coelomycete recognized as a widespread plant saprophyte, has been isolated from three diseased salmonid species in Washington and Oregon (Ross et al. 1975), and a previous outbreak was briefly described by Wood (1968) in Chinook salmon. Hatai et al. (1986) also described the condition of Phoma sp. Infection in farmed Ayu.

It has septate branched hyphae and young cultures on Sabouraud Dextrose Agar are light brown in color fading from light pink to black with age, with the formation of pycnidia producing hyaline unicellular conidia. Sometimes this septated phycomycete and other phycomycetes contaminate fish feed and can result in a granulomatous condition primarily of the kidney in a significant number of fish fed the particular contaminated batch.

In salmonid outbreaks, morbidity rarely exceeds 5% and the disease usually affects juveniles and juveniles. When disease incidence is high, fish swim abnormally and are unable to maintain their balance. They often have swollen blowholes with bleeding fins and skin lesions. The first internal lesions are limited to the swim bladder and are small white areas (1 to 2 mm) at the anterior end of the organ; The pneumatic duct area is likely to be the first to become infected.

In more advanced cases, the lumen fills with mycelium and the swim bladder epithelium becomes hyperplastic. The wall is quickly destroyed and adjacent internal organs are affected. There is a widespread acute inflammatory reaction or chronic granulomatous reaction, and petechiae and necrosis are found in affected internal organs. PAS and Giemsa positive hyphae are usually visible and are about 50–100 μm long and 2–3 μm wide. In Ayu-Brut, the fungus can be found in the swim bladder as well as in the intestines.

Pure cultures of the organism can be obtained by aseptically removing material from the abdominal cavity and plating on Sabouraud's dextrose agar.

The disease was described by Ross et al. reproduced. (1975) but the organism appeared to be only weakly contagious and the disease was found to be unrelated to diet.

6.14 ALGAE

primacy

Prymnesium parvum is a phytoflagellate from the order Chrysomonadinae that thrives in brackish water and has caused significant fish losses in Israel and other countries due to its extracellular toxins (Sarig 1971). The organism does not grow at salinities below 0.1%, but flowers develop over 3-5 days under optimal salinity, temperature and light conditions.

Early signs of toxicity in affected fish are schools of fish often violently jumping out of the water in the shallow waters of ponds. The fish will gradually slow down and eventually die. No gross pathologic signs are evident at autopsy, and death is caused by an osmotic imbalance resulting from exotoxin-induced increased gill permeability. Cationic activators are required for the toxin to be effective and a bioassay method using this principle is commonly performed by Israeli fish farmers using Gambusia spp. as a test fish.

Prymnesium control is carried out with ammonia compounds or copper salts.

6.15 ALGAE BLOSSOM

Seaweed blooms result from the rapid increase or accumulation of algae in an aquatic system. Typically, only one or a few phytoplankton species are involved. The number is often so large that the high density of their pigment cells leads to discoloration of the water. Although there is no officially recognized threshold, algae can be considered a bloom when their concentration

ranges from hundreds to thousands of cells per ml, but concentrations can reach millions of cells per ml. Algal blooms are often green but can also be other colors such as tan or red depending on the type of algae, which is why the common term red tide is often used.

Blooms can also consist of macroalgal species, not phytoplankton. These blooms are recognizable by large accumulations of algae that can wash ashore. Most algal blooms have limited effects on fish, but very dense algae can cause gill blockage and where there are sharp spines, such as in Agellate dinofl, short gill pathology can occur. Problems can also arise when there is a sudden slump in flowering, causing massive starvation of the water column. Algal blooms with toxic or otherwise harmful phytoplankton, such as frozen dinofl of the genera Alexandrium and Karenia, often produce ichthyotoxins in the water, resulting in significant fish kills. These blooms often take on a red or brown hue and, like other algal blooms discussed in this section, are colloquially known as red tide.

The Pfisteria group is a group of heterotrophic frozen dinofl associated with harmful algal blooms and fish kills. Sudden deaths of various species of fish in the Chesapeake Bay region of the United States in the 1980s, believed to be caused by Pfi steria piscicida, led to a serious human media illness known as "Pfi steria hysteria". and comprehensive surveillance of sea plankton by six of the eastern coastal states. Subsequent work showed that the Pfisteria clade is indeed distributed worldwide and that the majority if not all of the deaths from Chesapeake Bay Disease were caused by Aphanomyces invadans.

6.16 CYANOBACTERIA (BLUE ALGAE)

Meat spoilage and off-flavors due to certain types of blue-green algae are commonly reported in pond fish. Fatalities from these organisms can also occur and are associated with either toxin production or water deprivation due to massive deterioration from algal blooms (Rodger et al. 1994). Factors leading to the development of the flower include intensive fertilization and excessive waste of feed in fish ponds.

Control of blue-green algae is usually done by adding copper sulphate to the water, but this is an environmentally unsafe practice and great care should be taken when applying it to avoid rapid oxygen starvation from the decomposition of algae. Frequent use can lead to dangerously high levels of copper in the pond ecosystem.

CHAPTER 7

VIROLOGY AND CELL CULTURE

Research into viral infections in fish will be further expanded, particularly in the areas of novel viruses, comparative molecular biology of viruses, methods of virus detection, experimental studies on pathogenesis and taxonomy of viruses.

7.1 TYPE OF VIRUSES

Viruses are very small infectious agents that only multiply within the living cells of a host, using the host cell's machinery for their own reproduction. In the extracellular state, the virus particle or virion consists of nucleic acid surrounded by proteins and sometimes other macromolecular components. In this extracellular state it is metabolically inert. The virion is the structure through which the viral genome is transported from the cell in which it was produced to another cell where the viral nucleic acid can be introduced. Once in the new cell, virus replication begins; the virus genome is produced and other parts of the virus are also produced. The process of virus production is called infection. Other distinguishing features of viruses are their small size, ranging from 18 to 300 nm in diameter for spherical or globular viruses, and the fact that their nucleic acid (NA) is either DNA or RNA, but never both.

7.2 VIRAL MORPHOLOGY AND STRUCTURE

7.2.1 The virus

Virions vary greatly in size and shape. The shapes and relative sizes of the major families currently described in fish are shown in Figure 6.1. The viral particle or virion contains a genome (the viral nucleic acid) surrounded by a protein shell or envelope called the capsid. Some viruses have an extra envelope containing a lipid bilayer and proteins, usually glycoproteins. Although the virus membrane glycoproteins are encoded by the virus, the lipid is derived from the host cell membrane.

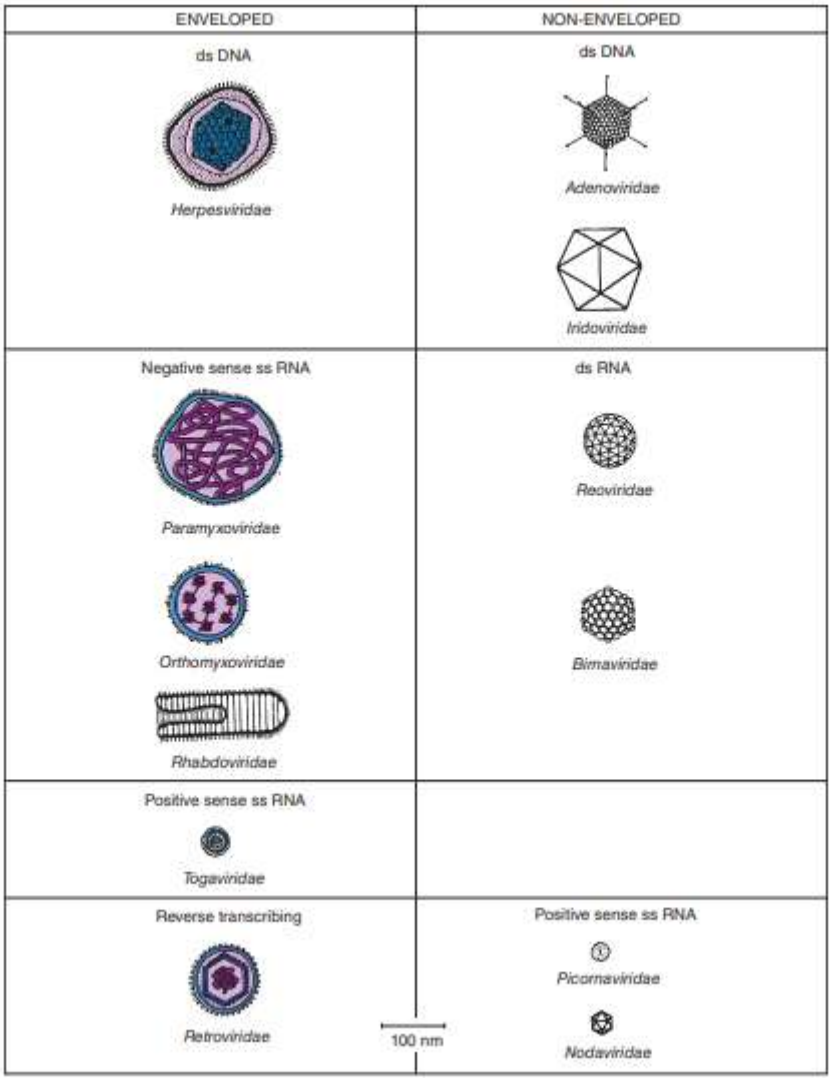


Figure 6.1 Fish virus morphology and nucleic acid content.

7.2.2 Nucleic Acid

The viral genome is either DNA or RNA. RNA viruses are unique in that they possess RNA genomes. RNA or DNA can be single or double stranded. In some viruses, the NA is segmented, while in others it is circular. Single stranded NA can be of positive or negative polarity. When the RNA is positive, synthesis of a complementary strand precedes synthesis of messenger RNA. If the RNA is negative (i.e. of complementary polarity), the messenger RNA must first be transcribed from it by a transcriptase carried in the virion.

The amount of NA in different viruses can vary widely. The smallest, the picornaviruses, have a relative molecular mass (M r) of about 2 × 10 6, while the M r of the herpesviruses is up to 150 × 10 6. In comparison, the Mr of mammalian cell nucleic acid is greater than 1012. The

molecular mass of the protein translated from the viral AN is about one-ninth the Mr of the AN (e.g., a picornavirus with an RNA of Mr 2× 10 6 encode a large protein of Mr 250,000 which would then be cleaved into several smaller proteins). These proteins would make up the viral structural proteins and enzymes.

The protein shell The shell consists of morphological units called capsomeres, which can be arranged according to three types of symmetry: icosahedral, spiral and complex.

7.3 ICOSAHEDRAL SYMMETRY VIRUS

An icosahedron is a regular polyhedron with 20 equilateral triangular faces, 12 vertices, and 30 edges (Figures 6.2 a, 6.2 b, 6.2 c, and 6.2 d). The capsomeres are arranged in equilateral triangular facets to form the capsid. The capsomeres themselves consist of subunits, each of which is formed from one or more polypeptides.

Virus with Helix Symmetry Here the virion is a long rod, the capsomeres are arranged around a helix of NA (Figures 6.2e and 6.2f). Many plant and bacterial viruses take this naked form. However, all vertebrate viruses with nucleocapsids with helical symmetry are enveloped, but only a minority of those with protein envelopes that exhibit icosahedral symmetry are enveloped. These single enveloped icosahedral viruses contain DNA, not RNA. The envelope is acquired by the virion during its release by budding from the endoplasmic or cytoplasmic nuclear membranes. Envelopes structurally resemble cell membranes and comprise a lipid bilayer containing transmembrane viral glycoproteins. Glycoproteins have glycosylated ends that radiate outward, and they have hydrophobic end caps embedded in lipids. The shell has an inner protein layer that anchors the glycoproteins. Glycoproteins are arranged in groups of 2 to 4 to form surface protrusions called spikes. Depending on the family, there can be 100 to 1000 spikes per virion.

7.4 VIRUS WITH COMPLEX STRUCTURE

The nature of the symmetry of some families (e.g. retroviruses) is even more complex. The structure of retroviruses is noted in the section on group characteristics of fish retroviruses.

7.4.1 Enzymes present in the virus

Some viruses require enzymes that are lacking in the host cell. In such situations the enzyme is present in the virus (e.g. reverse transcriptase of retroviruses). Sometimes a protein has a dual role (e.g. as an enzyme and as a structural protein).

7.4.2 Viral Antigens

Viruses can contain four to 100 structural proteins, many of which are recognized as being immunogenic, ie capable of inducing an immune response (antibodies and/or activated T cells) when appropriately administered to a living animal. Antigens are substances (often the immunogen) that react with the antibody and/or receptor on the T cell. The antibody or T cell receptor does not react with the entire antigen but with specific parts of the molecule called antigenic determinants or called epitopes.

Antigenic drift occurs through gene mutation in the form of substitution or deletion of bases in the nucleotide sequence of viral AN, resulting in a change in the amino acid incorporated into a protein. If an amino acid change changes the character of a neutralizing epitope that determines the serotype of a virus, a new antigen and thus a different serotype can arise. The mutation rate of RNA genomes is about 1000 times higher than that of DNA genomes. In fact, RNA replicas do not have proofreading activities like those of DNA polymerases. There are also other DNA repair systems that can correct changes before they become irreversible.

7.5 VIRUS CLASSIFICATION

Viruses are currently classified into 95 unassigned families or genera (Fauquet, Mayo, Manilof, Desselberger & Ball 2005) based on (1) genomic properties, (2) virion structure, and (3) protein properties. The main criteria are presented in Table 6.1. Classification within families into genera is based primarily on genomic traits and sequences, protein chemistry, serology, and host cell tropisms. Criteria for defining a virus species as opposed to strains of a species are provided by Van Regenmortel et al. (1997). These include genome components, degree of identity across an entire genomic and non-coding sequence, reaction with key antibodies, tissue effects and host range, and most importantly, consistency among virologists!

Table 6.1 Main features of vertebrate viruses used in classification.

For the genome	For the virion
DNA or RNA	Size
Relative molecular mass (M_r)	Shape
Single stranded (ss) or double stranded (ds)	Relative mass (M_r)
Polarity + or − (if ss)	Symmetry of capsid
Linear or circular	Number of capsomeres for icosahedral viruses
Polycistronic or segmented	Enveloped or not
	Cross-section architecture (e.g. spikes, projections, numbers and appearance of internal layers)
	Number of proteins and nature (i.e. structural and/or enzymic)
	Nature of enzymes
	Molecular weight of proteins

The cycle underlying acute infections in fish or other animals is demonstrated by inoculation of an appropriately selected tissue culture monolayer at a multiplicity of infection of approximately 1:10 (infectious units: tissue culture cells) or less. Adequate time is allowed for virus to attach to the cells, excess inoculum removed, growth medium added, and the culture incubated at an appropriate temperature. If the infectivity is tracked over the next few hours, a picture similar to that in Figure 6.3 emerges.

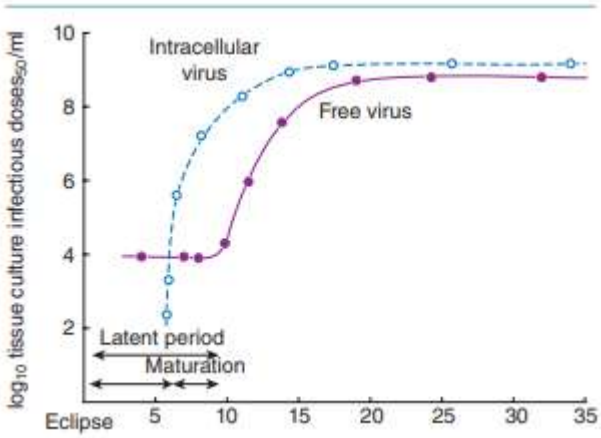


Figure 6.3 Idealized growth cycle of fish virus in tissue culture. The new infectious virus begins to mature at the end of the eclipse phase and accumulates intracellularly until cell lysis.

The new infectious virus begins to mature at the end of the eclipse phase and accumulates intracellularly until cell lysis. Initially, less virus is recovered than vaccinated and this is known

as the eclipse phase. After the eclipse phase, new intracellular viruses appear in logarithmically increasing amounts. The virus can then be released as free infectious particles. Eventually, virus production decreases. Most of the virus can remain attached to the cell, or alternatively, when the cell dies, most of it can be released.

The various stages of the viral replication cycle of a DNA virus are illustrated by a simplified diagram (Figure 6.4). After attachment, the virion is internalized by the cell and uncoated to reveal the genome, and the "early" viral genes are transcribed into messenger RNA. Early translated gene products are of two main types: proteins that (1) turn off host cell NA and protein synthesis, and (2) regulate expression of the viral genome and enzymes necessary for viral replication. After the "early" stage, the "late" viral genes are transcribed into late proteins that form the structural components of new virions. These can then undergo cleavage and glycosylation. Icosahedron virion assembly occurs in the nucleus or in the cytoplasm, depending on the attachment virus family. Enveloped viruses are completed as they bud through cell membranes. A representation of the retroviral reproduction of a budding virus is shown in Figure 6.5.

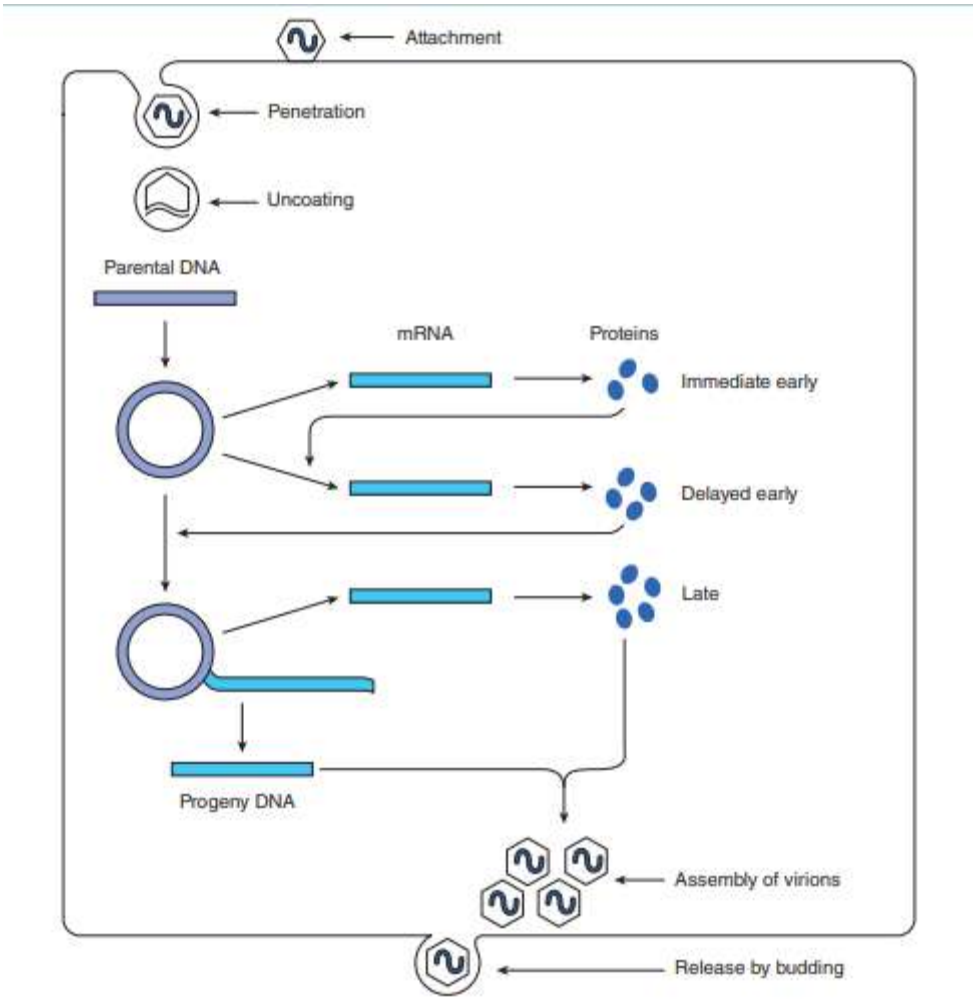


Figure 6.4 Stages of propagation of a DNA virus.

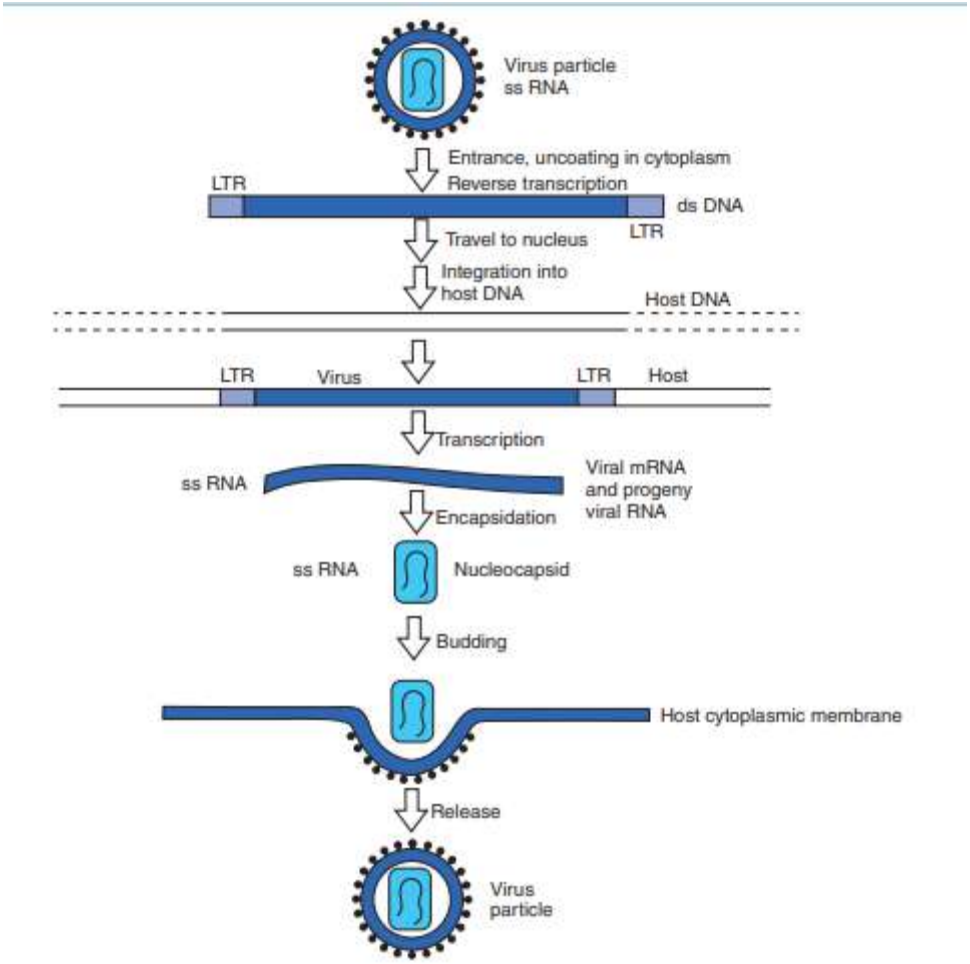


Figure 6.5 Stages of propagation of a retrovirus. LTR = long end repeat.

7.5.1 Appendix

Binding is a crucial first step in infection. Tight binding requires the presence of receptors for the virus on the plasma membrane. There is often a high degree of viral specificity for a host receptor, but some viruses are sometimes opportunistic and can share a common receptor. Not all cells or even all cells of a host organ necessarily have receptors, which may explain the often observed tissue tropism of viral diseases and their characteristic pathology. Conversely, the virulence of some viruses may correlate with decreased attachment to host cells, as the virus is less easily eliminated by phagocytes, allowing its spread to target organs. Even if a cell carries receptors, this does not necessarily mean that it will be infected, because after attachment the virus has to be detected and the replication cycle started.

When cells from a host organ are trypsin-dispersed and cultured in vitro, large changes in viral susceptibility can occur. Trypsin can digest the receptors, rendering the cells at least transiently

resistant until new receptors are generated, or alternatively, the receptors can be unmasked by trypsin digestion, allowing a viral growth cycle.

7.5.2 Intrusion

At least three different entry mechanisms are recognized, but their relative importance is unclear. They are endocytosis, fusion and translocation.

1. Endocytosis. "Most cells are capable of receptor-mediated endocytosis, a process commonly employed for the uptake of essential macromolecules. Visually, the process is similar to phagocytosis and pinocytosis, in which a small portion of the cell membrane forms an indentation with the virus bound to the receptor. The pit is pinched to form an inner vesicle that penetrates the cytoplasm and fuses with a lysosome to form a lysosomal vesicle. In some viruses, the viral envelope fuses with the lysosomal membrane and the viral core capsid is then ejected into the cytoplasm."

2. Fusion with the plasma membrane. "Enveloped viruses can fuse with the cell membrane by possessing glycoprotein and consequently release their nucleocapsid directly into the cytoplasm."

3. Translocation. "It is likely that some non-enveloped icosahedral viruses can pass directly through the plasma membrane."

Stripping Little is known about the details of this event and it is likely that the stripping mechanisms of DNA and RNA viruses are different. It is believed that viruses fusing with the cell membrane or lysosomal vesicle do not become coated. Some work suggests that icosahedral viruses can detach due to their attachment to the cell membrane. The transport mechanism of viruses that replicate in the cell nucleus is unknown.

Nucleic Acid Replication Naked viral nucleic acid encodes messenger RNA to make viral protein on cellular ribosomes. The viral nucleic acid also encodes a new viral nucleic acid that combines with the capsid protein to form the nucleocapsid. Viral genomes can have one of seven different forms of NA and therefore adopt as many NA replication strategies (see Carter & Saunders 2007). Replication can be promoted by primers and promoters and a variety of enzymes including polymerases, replicases and ligases. The seven major classes of the viral

genome are recognized by the NA type, whether they are double or single stranded in nature, and the polarity of the genome with respect to mRNA.

7.5.3 Transcription

Every genome class needs to replicate the viral protein, and for that viral mRNA is needed. Baltimore (1971) recognized and classified for the first time six of the mechanisms of transcription of NA virus genomes into mRNA (Figure 6.6); a seventh mechanism was later discovered. Some variation exists within the seven major classes, as some viral genomes have a single polycistronic chromosome, while others are segmented into molecules that may or may not encode a single gene. Messenger RNA transcribed from polycistronic genomes may or may not subsequently be cleaved enzymatically, with or without splicing, into gene-length mRNAs for translation into individual proteins. Splicing appears to be restricted to DNA viruses that replicate in the cell nucleus, as well as to retroviruses and orthomyxoviruses. Splicing regulates gene expression by producing a variety of mRNA species from one RNA transcript. Furthermore, the genome of some viruses can be read in different ways to encode different proteins, another remarkable technique for the economics of virus architecture.

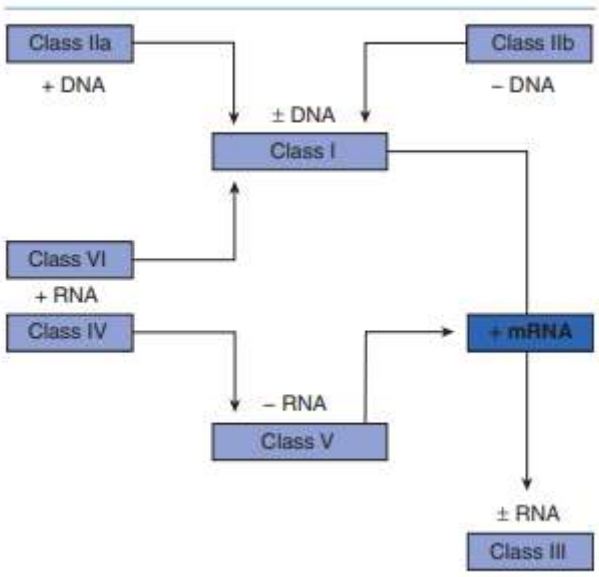


Figure 6.6 Baltimore classification of viruses based on the mechanisms of transcription of viral NA into mRNA.

Translation Viral mRNAs bind to host ribosomes in the cell's cytoplasm and are translated into proteins in the same manner as eukaryotic mRNAs. Proteins translated from early viral gene

transcripts include enzymes and other proteins thought to have important regulatory functions in transcription control. Viral proteins are structural proteins designed to be incorporated into new virions, but some also function as regulatory proteins that can stop the transcription or even translation of early cellular or viral genes.

Newly synthesized viral products must travel to sites where they are needed for assembly, for example, herpesviruses replicate in the nucleus, and although viral proteins are synthesized in the cytoplasm, they must be transported back to the nucleus. In the case of glycoproteins, the polypeptide backbone is translated on membrane-bound ribosomes (ie, the endoplasmic reticulum); Various modifications including acylation, proteolytic cleavage, sugar addition and modification occur sequentially as molecules move from vesicles to the Golgi apparatus and then to the plasma membrane.

Viral Assembly In simple spherical viruses with icosahedral symmetry, structural proteins can spontaneously assemble into capsomeres, which in turn self-assemble into procapsids around the NA core. Fortunately, many particles may not contain NA, resulting in void particles. Assembly of DNA viruses occurs most frequently in the nucleus and of RNA viruses in the cytoplasm. Assemblies of individual spherical viruses or empty procapsids can form paracrystalline lattices, which can sometimes be seen under a light microscope as a form of inclusion bodies.

Enveloped viruses always contain glycoproteins of viral origin. Viral glycoproteins normally migrate from ribosomes to the host cell membrane via the endoplasmic reticulum, with individual glycoproteins aggregating into characteristic spike shapes. During assembly of an enveloped virus, the nucleocapsid aligns under the plasma or nuclear membrane and multivalent attachment of many glycoprotein spikes occurs. Surrounding the nucleocapsid is a protein layer embedded in the lipid membrane. This process forces the nucleocapsid, surrounded by spikes of viral glycoprotein, through the host cell membrane to form the distinctive bud on the cell.

Virus Release Non-enveloped viruses are released when the cell dies and breaks down, processes that result from the shutdown of cellular metabolism caused by viral products. Enveloped viruses are released through budding, which is often a gradual process.

Defective replication Many released virions are non-infectious, which can result from an incomplete genome, incomplete formation of their proteins, or denaturation between release and assay.

Many viruses, particularly RNA viruses (eg, infectious pancreatic necrosis virus or IPNV), produce a defective virion known as a defective interfering particle (DI) (Huang & Baltimore 1977). The DI particle has the following properties: first, the genome is defective and therefore non-infectious; second, the particle can only replicate in the presence of a standard infectious virus that serves as its helper; third, the DI particle interferes with the replication of the infectious virus by slowing down its rate of multiplication; and fourth, DI particles often preferentially multiply at the expense of the infectious virus. In cultured cells, the interference acts intracellularly at the level of viral replication. Possible reasons for the DI enrichment are less time needed to synthesize the shorter lengths of NA in DI particles; their NA is used less frequently as a template for transcription and their NA has increased affinity for viral replicase. DI particles are believed to play an important role in persistent viral infections.

Latency In this mode, the virus can persist over time without replicating itself as a non-replicating virion, either as a viral genome in the form of an episome, or as proviral DNA that integrates into the host cell's chromosome. Integration is a distinctive feature of herpesviruses (e.g. catfish channel virus or CCV). Latency is important because in a stressed host (eg, when the immune response is suppressed by high levels of corticosteroids), a cycle of viral replication can be initiated with the possibility of clinical disease. Recurrent episodes of clinical illness can be a feature of these latent infections.

7.5.4 Processing

A concomitant result of the integration of viral NA into the host genome is that the cell can transform with a gross change in growth characteristics. Viruses that cause such transformations are called oncogenic viruses, and Oncorhynchus masou virus (OMV) is an example of this role in fish.

7.6 EFFECTS OF VIRUS INFECTION IN THE CELL

These can be subdivided as follows:

1. Early signs of visible changes such as cloudy swellings observed in histological preparations.

2. Irreversible changes leading to death. This is called the cytopathic effect (CPE).

3. Irreversible effects leading to the loss or deterioration of a specific function (e.g. endocrine secretion).

4. Transformation to a neoplastic state (e.g. OMV).

5. Infection persisting after (1) or (3). Viral NA can be integrated into the genome with intermittent shedding. Examples are given in several families of viruses that infect fish.

Regardless of the end result, various changes in the cell picture can be observed during the initial phase of viral infection:

1. Areas of the cell with altered staining properties as seen by histology.

2. Aggregates of viral antigens can take on characteristic shapes called inclusion bodies. They can be seen on histological sections stained under a light microscope (e.g. lymphocyst virus).

3. Fusion of two or more cells into a multinucleated giant cell or syncytium.

At the molecular level, viruses that cause ECP likely shut down macromolecular synthesis in the host cell, thereby preventing protein and NA synthesis. Accumulations of viral capsid proteins can have inhibitory effects on enzymes in host cells distant from the site of infection.

7.7 EFFECTS OF VIRUS INFECTION ON HOST FISH

As soon as the virus spreads beyond a few host cells, a variety of non-specific and host-specific defense reactions are triggered. Depending on how effective these responses are in containing viruses and minimizing damage to host cells and organs, the result is either (1) clinical disease with morbidity that can be recognized by the clinician or histopathologist; or (2) infection without symptoms, representing a silent infection. The infection itself can therefore only be determined by detecting the presence of viruses or viral antigens, or subsequently by other laboratory tests such as detecting anti-viral antibodies.

Usage has associated the term virulence with the characteristics of a virus that lead to disease. Determination of virulence can be made by (but not limited to) measuring mortality, morbidity, tissue damage, decreased growth rate, or loss of cell function.

The result of infection can be that:

1. the host shows no clinical disease and is shedding the virus;

2. There is no clinical disease but the infection persists (carrier status);

3. the host develops clinical disease and dies;

4. the host develops clinical disease, recovers, and sheds the virus; or 5. the host recovers from the clinical disease, but the infection persists with no clinical manifestations (the carrier state).

With successful infections and regardless of the outcome, the virus is shed either over a period of time or possibly continuously. The virus can be shed in feces or urine and from epithelial surfaces (eg, gills and skin).

Persistent infections

Viruses can persist for long periods of time, possibly the life of the animal (Fenner et al. 1974). These infections are difficult to satisfactorily classify, but for the sake of simplicity three broad categories can be distinguished, recognizing that there is some overlap between the categories:

1. Persistent infections with possible one or more acute episodes of clinical illness between which viruses are usually undetectable (e.g. salmonid IHN virus).

2. Persistent infections in which the virus is still detectable and is shed frequently, but the clinical disease is absent or only manifested by physiological disorders (eg, salmonid IPNV).

3. Persistent infections with a very long incubation period followed by slowly progressive clinical disease that is always fatal (ie, slow infections). CMS (see family Totiviridae, tentative placement) has many characteristics of slow infections with potential for inducing myopathy stress.

Little is known about the important factors in the establishment of a persistent infection in fish. From mammalian and avian studies it is postulated that the following is relevant:

1. Growth in sheltered areas, e.g. B. cells of the nervous system, lymphatic tissue and epithelial surfaces.

2. Integration of the viral genome into the host cell genome (e.g. CCV in catfish).

3. Non-immunogenicity and non-elicitation of non-specific host protective factors (ie no antibody response and no interferon production).

4. Non-neutralizing antibodies that protect the virus from neutralizing antibodies and cell-mediated immunity.

Age and resistance to viral infections

The response to viruses differs dramatically in many animals and fish are no exception, with the effects of most viral infections often being most dramatic early in life. The first few weeks after hatching are a period of rapid physiological change. At this stage, young fish may experience severe generalized clinical disease, while older animals with a similar initial exposure may experience only asymptomatic infection. Inadequate maturation of protective mechanisms such as the inflammatory response and immune response can be one of the causes of the susceptibility of young fish to infections.

7.8 PRACTICAL ASPECTS OF FISH VIROLOGY

7.8.1 Diagnosis of viral diseases

Culture

By far the most common approach to diagnosing fish viral diseases is to grow the virus in a monolayer tissue culture. Apparent cytopathic effects (CPE) are the expected consequence of virus growth in the cell monolayer and form the basis of a positive initial diagnosis. However, culture of a virus should always be confirmed by a second diagnostic procedure.

Primary monolayer cell cultures are prepared by subjecting small pieces of appropriate fish host tissue (e.g., head, kidney, gonad, or embryo) to partial proteolytic digestion and then adding the released cells to growth medium in glass or plastic vessels. In any case, some cells can grow on the surface and form a layer. This leaf can be removed by trypsin/EDTA digestion, divided and seeded again in other containers. Primary cultures are usually slow-growing during the first few passages, but after 10–20 passages the cells undergo more rapid mitotic cell division and may undergo several hundred passages. Such established cell lines are widely used for virus cultivation.

Monolayer cultures are grown in complex tissue culture media in special containers made of glass or non-toxic plastic. For maximum cell yield, vessels with large surface areas are used on which to grow the monolayers. Tissue culture medium is a sterile isotonic solution of essential inorganic ions, amino acids, glucose as an energy source, antibiotics, pH buffer and color indicators, supplemented with serum. Complex growth factor requirements are most easily met by serum (usually fetal calf serum or bovine serum) at 10% for growth and 2% for maintenance.

Most fish viruses produce obvious degenerative changes in cell culture that can be detected by live culture microscopy (e.g., rounding, indentation, necrosis, syncytial formation and detachment). Different viruses cause different types of EPC and this can be used for initial diagnosis, but final diagnosis must be made using other methods. Typically, a confirmatory diagnosis is performed using a specific antiserum in an infectivity neutralization test, an immunostaining method for viral antigens in suspect monolayer cultures, or an enzyme immunoassay (ELISA). For serological neutralization, infectious material from tissue cultures is first titrated with CPE to determine the number of infectious units. A dilution containing approximately 100 to 200 infectious units is mixed with a series of two-fold dilutions of specific antiserum and infectivity reassessed. The absence of CPE production indicates a specific neutralization reaction. For antigen detection, a specific antiserum to which either a fluorescent dye or an enzyme (e.g. peroxidase) has been bound is reacted with the tissue culture cell monolayer showing CPE and the excess antiserum is eliminated. The covalent attachment of enzymes to antibody molecules creates an immunological tool with both high specificity and high sensitivity. The technique is called ELISA for enzyme immunoassay. Virus-positive samples are obtained by retention of either the fluorescent dye, as detected by UV microscopy, or the ELISA system, as detected by a colorimetric assay with an appropriate substrate.

microscopy

Light microscopy cannot resolve most individual virus particles because they are too small. However, some of the larger iridoviruses (300 nm in diameter) can be detected as particles in stained swabs (e.g. viral erythrocyte necrosis virus). Virus inclusion bodies, which are induced by certain fish viruses, can be observed under the light microscope in histological sections and specially stained smears.

Electron microscopy can also be used to visualize viral particles and determine their morphology. This approach can be important when investigating a new viral disease of unknown etiology. Due to the complexity of sample preparation, the cost of specialized equipment and skills, and the lack of an absolute guarantee of success, this approach is best suited for characterizing the morphology of a virus isolate grown in culture tissue rather than as a routine method to screen for new viruses in lesion material.

Detection of specific viral components

These can be detected directly in the host tissue if enough viruses are present. The methods are based on the detection of viral structural proteins or viral NA.

Detection of viral proteins

Antiserum to whole viruses may react with a suspicious tissue smear, histological tissue section, or tissue culture monolayer of infected cells. The antiserum can either be labeled directly with a fluorescent dye (e.g. fluorescein) and detected by microscopy (fluorescent antibody technique or FAT), or with an enzyme, the presence of which is then the basis of the ELISA. An alternative approach is to use an unlabeled antiserum (e.g. rabbit antiviral antiserum) and then use a second labeled antiserum (e.g. fluorescein-labeled or enzyme-linked goat anti-rabbit). This alternative approach is more sensitive.

Polyclonal or monoclonal antibodies specific for a fish viral protein can be used in a similar manner. This approach may have additional sensitivity to discriminate a virus generally at the genus or additional subgenus level. A refinement called an immunoblot is a very sensitive method to detect the presence of a specific protein. Four steps are involved:

1. Separation of viruses from tissue culture material by centrifugation.

2. Separation of viral proteins on a polyacrylamide gel (PAGE) containing sodium dodecyl sulfonate (SDS).

3. Transfer of proteins from gel to nitrocellulose paper by electrophoresis.

4. Subsequent identification of the protein by labeled antibody. Protein blotting followed by protein identification is often referred to as the western blotting technique.

Detection of viral NA

Unique NA sequences exist within each virus family, between genera within a family, and even between isolates within a genus (e.g., from different geographic areas). The nucleotide sequence is so specific that hybridization analysis can be used in research to identify new isolates and for reliable clinical diagnosis. Automation has made DNA analysis routine in many laboratories. Automated DNA extractors, polymerase chain reaction (PCR) devices, DNA sequencers, and pulsed field electrophoresis devices to separate DNA segments are all used in fish virology today. A useful molecular tool is the nucleic acid probe, which is a key reagent in the in situ hybridization assay.

nucleic acid probes

Hybridization of the Probe to the NA of the Target Virus The NA detects the presence or absence of specific sequences associated with a specific virus. The probe is a single-stranded complementary synthetic oligonucleotide (cDNA) containing sequences unique to the virus. Obviously, at least the sequence of a portion of at least one gene of the virus must be known. The synthesized probe can be several kilobases long, but many synthetic sequences are around 20 bases long and are always very specific. The approach is to force the NA virus to form single-stranded molecules and allow the labeled oligonucleotide probe (enzyme, radiolabel, or fluorescent compound) to hybridize under appropriate conditions. The detection sensitivity is approx. 0.25 µg NA, i.e. 10 6 virions. This is not as sensitive as culture, but still useful (e.g. when the virus cannot be cultured).

Polymerase chain reaction

Based on DNA hybridization, PCR can multiply DNA molecules up to a factor of 10 9. As with probes, the PCR technique requires knowing the nucleotide sequence of part of a gene. Basically, the procedure is as follows:

1. Two short labeled cDNA probes or complementary primers for each end of the sequence are added in large excess to the heat denatured DNA.

2. As the mixture cools, the excess primers over the target DNA ensure that most strands anneal to one primer and not to each other.

3. DNA polymerase (or RNA-dependent DNA polymerase if an RNA viral genome was the initial target) extends the primers using the target strands as a template.

4. After an appropriate incubation period, the mixture is reheated to separate the strands. The mixture is then cooled to allow the primers to hybridize to the complementary regions of the newly synthesized DNA, and the entire process is repeated 20-30 times, resulting in a 10 6-9 increase in sequence target.

PCR can detect nanograms or smaller amounts of RNA and DNA and can replace culture in terms of sensitivity; It has been claimed to be able to detect between 1 and 10 virus particles/g tissue. Nested PCR further increases sensitivity by amplifying a smaller portion of the already amplified PCR product. Differential PCR uses a series of primers, with one primer common to the gene in all virions and the others each being specific to a particular group of viruses. It is used to distinguish between closely related viruses and its approach is similar to using panels of monoclonal antibodies (Mabs).

Real-time or quantitative PCR detects and measures ("real-time") cDNA or target DNA amplification during the exponential growth phase of the PCR reaction, the optimal point to analyze the data from the PCR reaction and precisely quantify the starting target DNA or RNA without the need for laborious agarose gel electrophoresis.

One of the primary methods employed in performing real-time PCR analysis involves the use of specific DNA probe sequences labeled with a fluorescent reporter dye and a quencher dye. Amplification of the target material is detected during the reaction by binding of the probe to a specifically amplified PCR product. As long as the probe is intact, the quencher dye will absorb any fluorescence emitted by the reporter dye. During the PCR, the cDNA or DNA is repeatedly denatured at high temperature, thereby separating the double strand, allowing the primers and the probe to hybridize. After binding, the probe is cleaved by the 5' nuclease activity of the DNA polymerase enzyme. This detaches the quencher from the reporter and produces fluorescence emission that increases proportionally to the amount of amplification and is expressed relative to a standard dye present in the sample. Another method used to perform real-time PCR analysis uses non-specific reporter dyes that bind to double-stranded DNA and produce fluorescent emission. As the number of double-stranded amplicons increases during the PCR reaction, the intensity of the fluorescent signal increases and can be quantified by cross-referencing with standard dilution.

Fish antibodies as an indicator of infection

This approach can be a useful non-destructive method for determining current or past infection in a population. Details on the procedure can be found in Chapter 4. However, experience with several fish species suggests that due to the apparent lack of an immune response in individual fish, no fish in an infected population can be considered free of infection due to the lack of antibodies.

7.8.2 Virus Inactivation

Knowledge of methods to inactivate viral infectivity is important for disinfecting fish culture systems and fish eggs, and for disposal of infected equipment and waste.

physical methods

temperature

Heat at 56°C for 1-2 hours inactivates most viruses, except for IPNV and Birnavirus, and less time is required at higher temperatures. At 4°C the loss of infectivity can be very slow and depends on the suspension medium. From -10°C to -25°C, infectivity survival varies from one virus to another and between strains of the same virus. At very low temperatures (e.g. −70 °C or lower), the survival rate is high.

radiation

Ionizing radiation (e.g. X-rays and gamma rays) will quickly inactivate the virus, as will short-wavelength light (e.g. UV). Direct sunlight also inactivates most viruses, especially if they've dried on a surface.

chemical methods

PH value

Most viruses are nearly neutrally stable (pH 6.4-7.4) and some are stable at low pH (e.g. pH 3 for IPNV). Below pH 3 and above pH 11, most viruses are quickly inactivated due to the strong denaturing effect of proteins. For this reason, 2% sodium hydroxide solutions are effective disinfectants on plastic, fiberglass, and concrete surfaces, but should not be used on metal, which is prone to severe corrosion. Similarly, quicklime (CaO) is an effective disinfectant for the surface of drained earthen ponds where it can be buried for more effective results.

Oxidizing chemicals

Halogen preparations, e.g. hypochlorite or iodine in aqueous solution or in the form of iodine tincture (KI + I 2), or in iodophor preparations, inactivate all viruses, provided their effect is not reduced by an excess of organic matter and the contact is made for a period of time maintain. Std iodophor preparations are less corrosive and less irritating and, importantly, also surfactants (i.e. detergents), giving them greater power of penetration into cracks and rough surfaces. Halogenated preparations, particularly iodophors, are widely used as general disinfectants for tanks, pipes, footbaths, car wheels, nets and other paraphernalia, although it should be emphasized that prior cleaning is essential for their effective use. Iodophors in diluted buffered form can also be used for surface disinfection of fish eggs.

Ozone is commonly “used in aquarium water treatment to inactivate viruses and bacteria. It is very efficient, but the initial equipment cost for large-scale production is high.

Persulfate compounds are another group of oxidizing chemicals commonly used in the laboratory to disinfect glass and plastic items.

alkylating agent

Formaldehyde and glutaraldehyde solutions inactivate the virus due to their ability to both crosslink proteins and penetrate and react with the NA of the cell nucleus. Their main use is in the manufacture of animal vaccines, but as a tissue preservative for histological material, they effectively destroy any infectivity they contain.

laundry detergent

They are used for cleaning rather than disinfection, although they inactivate enveloped viruses due to their lipid-dissolving effect. They can be mixed with compatible disinfectants (e.g. formaldehyde and glutaraldehyde) to increase penetration as an alternative to iodophor compounds. Amphoteric detergents based on alkyldiaminoethylglycine, which can be mixed, retain their cleaning properties in acidic and alkaline conditions.

7.9 VIRUS STORAGE

Infectivity can be stored indefinitely at -70°C or below. However, viruses associated with cell cultures may require the assistance of dimethyl sulfoxide (10%) and serum (10%) or even

glycerol (50%). Lyophilization (lyophilization) of the virus in fetal bovine serum (10%) and storage below 4°C is another successful preservation method.

7.10 PREVENTION AND CONTROL OF VIRUS DISEASES IN FISH

avoidance

Currently, the best way to prevent infection of farmed fish is to avoid getting the virus in fish stocks, water supplies, or equipment and materials that have been in contact with infected material. If the virus is to be absent, this means that all additions of fish and fish eggs to virus-free stocks must themselves be virus-free. The water supply should be virus free (e.g. artesian water, spring water, filtered or otherwise treated water). If surface waters are to be used untreated, they must either be free of fish or the fish populations they contain must be virus-free. Once this virus-free status has been achieved, all fish supplements in the watershed should also be virus-free. Other approaches to prevent viral diseases are as follows.

vaccinations

Live vaccines, so-called attenuated vaccines, contain virulent or very weakly virulent virus strains that trigger a protective immune response in the event of intentional infection. Because live vaccines are administered by bath and only very small amounts are involved in infection, they have great advantages for fish farming applications. Experimental live vaccines have been reported for IHNV, VHSV and CCDV (see single family text, this chapter). However, such infections result in infected fish releasing viruses into the non-farm aquatic environment. Regulators responsible for approving vaccines should rest assured that these virus strains are unlikely to revert to more virulent forms. So far, none has been approved anywhere in the world.

Killed vaccines contain non-infectious viruses. They are produced by growing virulent viruses, followed by inactivation, usually by chemical means (e.g. formalin), but retaining the immunizing properties of viral antigens that confer protection. Possible vaccination routes are injection, oral administration with food" and bath.

Genetic engineering offers two alternative approaches to the production of dead vaccines. In the first case, the viral gene encoding an important protective polypeptide antigen is incorporated into the genome of a bacterium by recombinant DNA technology, where it is

expressed. This is an inexpensive method of producing a so-called subunit or recombinant vaccine. The second approach is to directly inject the virus gene, suitably conditioned for expression, into the fish. This is called a DNA vaccine. Research on the efficacy of experimental DNA vaccines made good progress between 2000 and 2010, and a DNA vaccine against IHNV was approved in Canada for use in the aquaculture industry (see the Rhabdoviridae and IHNV sections of this chapter). The use of DNA vaccines is not without consequences for the environment and fish health. The possible adverse consequences of using DNA vaccines were discussed in a large study by Gilund et al. (2008). The conclusion was that more research was needed on the stability of plasmid DNA (pDNA), unintended immunological effects, and the actual integration of pDNA into the host genome. For killed and subunit vaccines, carrier status is likely to occur after exposure to the virus and therefore all fish exposed to the same waters are at risk unless vaccinated.

chemotherapy drugs

Although a limited number of human viral infections can now be treated with chemotherapeutic agents, there are currently no commercial antiviral chemotherapeutic treatments for" fish.

Genetic selection and breeding for resistance

Breeding resistant strains of fish is one possible approach to disease control, and reduced susceptibility to individual diseases has been reported for some strains of cultivated species. Breeding programs based on crossing strains with different resistances and measuring the level of inherited resistance are well established in Scotland and elsewhere (Houston et al. 2009). However, the resistance mechanism must also be clarified, since so-called resistant animals are actually tolerant and can become persistently infected if infected. Such a consequence can lead to further spread of the pathogen to susceptible animals.

well-being and hygiene

In aquaculture, fish stress is most commonly caused by high stocking density and poor water quality. These are important factors that trigger infectious diseases and/or increase their effects. Ensuring a minimum of stress is therefore an important element in any disease prevention and control. Good hygiene practices (e.g. cleanliness of tanks and fish handling equipment, disposal

of cadavers and off-site waste treatment) are other important factors in controlling viral diseases.

7.11 EPIZOOTIOLOGY OF VIRUS INFECTIONS AND DISEASES IN FISH

Epizootiology can be defined as the study of the spread and determinants of infection or disease in host and vector species. This implies that infection is not randomly distributed among species or populations, but that groups within a species or population differ in how often they become infected or carry different pathogens and whether clinical disease results. Knowledge of this uneven distribution and virulence of pathogens can be used to study transmission routes and infection reservoirs and to identify information for prevention and control.

transmission

Lateral spread, in which infection occurs between individuals in a population, occurs through three main routes of virus entry into fish: skin, gill tissue, and intestinal tract; Eye, nostrils and barbels are other possible secondary pathways. In vertical transmission, the virus is transmitted directly from parents to offspring from one generation to the next via germ cells.

The importance of specific modes of transmission is presented in the following discussion of each viral disease. Human activities to move fish stocks for aquaculture and stocking of rivers and lakes, which often involve the introduction of new species or new strains of existing fish species into an area, should be considered as a potentially important factor in the spread of both virulent strains of existing viral diseases and new viral diseases outside of their current geographic boundaries.

infection reservoirs

Viruses are found in both wild and farmed fish populations, and fish viruses can also be transmitted by other animals. Many virus pathogens of fish diseases are only known from their effects on farmed fish. We know little about their distribution in wild fish populations. In this section of the chapter, prevention could only be considered to a very limited extent, since a lot of essential information is missing. Much of the current advice on preventing viral diseases on fish farms is to keep fish under conditions that preclude the presence of viruses, but these conditions are often impossible to meet. The most commonly used bodies of water are rivers,

lakes, drains, and seawater, all of which contain many wild aquatic species with unknown disease reservoir potential.

The presence of viral diseases in fish farms leads to the escape of infected fish and the release of viruses (especially in animal diseases) into the aqueous medium. The impact of the virus outbreak on wild fish populations is largely unquantified. However, this knowledge is important both for fisheries, since their stocks could eventually be reduced or certain species would benefit from selective advantages, and for aquaculture, where knowledge of pathological events in wild populations can allow problems to be foreseen and thus avoided or to reduce farms.

7.12FISH VIRUS INFECTION – DNA VIRUS

7.12.1 Iridoviridae

The group gets its name from the iridescent Tipula virus, which grows in the hemocoel of the leather jacket (Tipula sp.) and makes the fly iridescent. In insects, viruses are non-enveloped and ether-resistant and grow in very high concentrations, forming polyhedra and paracrystalline networks. Vertebrate iridoviruses are generally larger and may have an envelope derived from the host plasma membrane. This envelope may not be critical to infectivity, but it does enhance it (Aubertin 1991). Iridoviruses are large, isometric viruses with icosahedral symmetry, 120–350 nm in diameter, and consist of a spherical nucleoprotein core surrounded by a membrane composed of protein subunit-modified lipids. The genome consists of a single linear double-stranded DNA molecule, M r 1.05 - 2.75 × 10 9 (Chinchar et al. 2005). Transcription and DNA synthesis are nuclear and virion assembly is cytoplasmic. In fish, three of the five genera of iridoviruses are represented, namely Lymphocystis, Ranavirus (from Latin rana for frog, as the genus also includes amphibian viruses) and Megalocytivirus (from Greek for enlarged cell).

7.12.2 Iridovirus diseases of fish

There have been many reports of iridovirus-induced disease in fish. Ahne (1994) divided swimming pool iridoviruses into three groups according to their pathogenicity, morphology and antigenicity, namely:

1. Viruses associated with hypertrophy of connective tissue cells (lymphocystosis virus).

2. Viral agents that cause epizootic hematopoietic necrosis (EHNV)-like disease.

3. Viral agents associated with erythrocyte necrosis (VEN).

Each group shows tropism for different host tissues (ie, connective tissue, endothelial tissue, and erythroid tissue for Lymphocystis, EHNV, and VEN, respectively). Taxonomically, Lymphocystis viruses belong to the genus Lymphocystivirus and EHN viruses to the genus Ranavirus. Since Ahne's review, which was superseded by that of Essbauer and Ahne (2001), iridoviruses of the genus Megalocytivirus, which cause enlarged cells in a variety of tissues, have been extensively studied and characterized. The best-studied species are red sea bream iridovirus (RSIV) and infectious spleen and kidney necrosis virus (ISKNV). This group therefore represents a fourth virotropism, "viral agents causing enlarged cells in a variety of tissues", as major pathogens for the aquaculture industry in the Far East (e.g. Taiwan) (Chao et al. 2002). In addition, a fifth virotropism could be added, represented by the white sturgeon iridovirus epitheliotropism for epithelia (see below). The viruses responsible for VEN, erythrocyte necrosis virus (ENV) and white sturgeon iridovirus, have yet to be placed in a genus within the family.

7.13 GENUS LYMPHOCYSTIVIRUS

7.13.1 Lymphocyte Virus Disease

Fish lymphocytic disease is a well-known viral infection presenting as nodular skin lesions and is known to affect a wide variety of freshwater, brackish and saltwater fish. It was one of the first viral diseases of fish to be described when Lowe (1874) reported it in the European Flounder. Evidence of a viral etiology appeared only in the EM studies by Walker (1962) and virus isolation in BF-2 centrachid cells by Wolf et al. on. (1966). Anders (1989) listed the disease as occurring in 141 teleost species and there have since been further reports in other species.

pathology

Small cream-colored nodular lesions are seen on the skin (Figure 6.7) and fins (Figure 6.8), and similar nodules occur inside the mesentery and peritoneum (Figure 6.9). Each nodule consists of a single cell infected with Lymphocystis virus or a lymphocyst visible to the naked

eye, up to 1 mm in diameter. More commonly, multiple hypertrophied cells occur singly or in tumor-like, raspberry-like clusters.

The cytological course of a Lymphocystis infection was described in bluegill by Dunbar and Wolf (1966) and in plaice by Roberts (1976). Although the timescales for lymphocyst development and regression are very different (28 days at 25°C for Bluegill versus 3 months at 10°C for plaice), certain well-defined stages can be identified:

1. Fibroblast-like cells stop dividing but continue to grow and massively enlarge, showing basophilic cytoplasm and prominent nuclei and nucleoli.

2. As the cell enlarges, cytoplasmic inclusions surrounded by clear, halo-like areas are visible. These inclusions stain DNA (i.e. Feulgen and Acridine Orange positive).

3. In the middle of maturation, a hyaline capsule becomes clearly detectable by hematoxylin and eosin staining, and cytoplasmic inclusions become more fragmented in a basket-like pattern.

4. A degenerative phase follows. Nuclei and nucleoli appear condensed and ill-defined. The inclusions remain near the periphery and the hyaline capsule appears degenerate. Macrophages and scavenger cells concentrate near the degenerating lymphocysts and can invade the lymphocysts. Degradation of the lymphocyst can lead to new de novo infection of the neighboring fibroblasts. Otherwise, the virus does not appear to be released until the lymphocyst is eliminated and then lysed, releasing the virus into the environment.

In plaice, a pronounced inflammatory response occurs 3 months after infection with evidence of a cell-mediated immune response (CMI) and anti-precipitin antibodies. In early lesions, macrophage infiltration into the lymphocyst is weak, but by 6 weeks post infection a 30 μm inflammatory collar composed mainly of epitheloid cells surrounds the cyst. At the late lesion, at 3 months, this collar has a vascular stroma supplied by a network of capillaries and plasma cells, and lymphocytes are also present.

Russell (1974) studied the histopathology of lymphocytic lesions from wild plaice and plaice and found that there were marked differences in the morphology of lesions at different locations. The response to serum precipitation was much more frequent in wild plaice than in plaice. There is also a marked seasonal prevalence, with peaks in summer for some species and

winter for others. The lesion is problematic for the culture of valuable species such as Japanese plaice, as infected fish become deformed and exhibit poor growth and anemia with marked losses (Iwamoto et al. 2002).

virology

Lymphocystis disease virus (LDV) is an isometric particle that varies in size from 130 to 380 nm across the peaks, depending on the origin of the fish host. The virus consists of a bilayered capsid and a nucleus that appears filamentous and has helical symmetry (Madeley et al. 1978). The cell nucleus is surrounded by a membrane-like structure that is clearly visible in the decaying virus. Negative staining of the decaying virus shows that the electron-transparent outer layer of the capsid consists of knobs, possibly attached to the inner layer of the capsid by spines (Figure 6.11). Samalecos (1986) showed that treatment of LDV with papain prior to staining reveals a lattice structure of capsomeres, presumably by removing the outer capsid layer. Robin and Berthiaume (1981) observed that Bluegill (Leetown NFH strain) LDV particles had two sizes per EM. Large 300-350 nm particles were associated with recovery of infectivity and small 100-150 nm particles, presumably maturing virions, were without infectivity.

7.15 MOLECULAR DETECTION AND COMPARISON OF VIRAL STRAINS

PCR coupled with a slot blot hybridization method proved to be a sensitive method for detecting 2.5 ng of viral DNA in asymptomatic carrier fish (Cano et al. 2006). Although this method is comparable to EPC in terms of sensitivity with immunoblotting and virus culture in carrier seabream, the time saving compared to virus culture was very significant. Slot blot PCR coupled with tail fin samples allowed rapid molecular confirmation of LDV without killing fish.

At least two strains of Lymphocystis virus can be distinguished. Based on genome analyzes of Pleuronectidae lymphocystis disease (LDV) viruses, Anders and Darai (1985) showed that there are different species or strains of viruses infecting different species within the family, LCD-1, found in the flounder Platichthys flesus and the plaice Pleuronectes platesa, and LCD-2 in blob Limanda limanda (Darai et al. 1983; Schnitzler & Darai 1993). This finding confirmed Russell's (1974) observations of differences in pathology in different loci and species.

The complete DNA sequence of plaice lymphocytic disease virus (LCDV-1) was published by Tidona and Darai (1997), these authors reporting a genome of 102,653 bp in length with 195 open reading frames. Zhang et al. (2004) then reported the genome sequence of LDV from cultivated plaice in China (LDV-C). Since the genes for the major capsid protein (MCP) of the two strains were only 87% identical, it was assumed that the two strains represent different species within the genus Lymphocystivirus.

Further studies on a variety of isolates within the genus identified six genotypes (Hossein et al. 2008), with LCDV-1 representing genotype GI and LCDV-CG-II. Kitamura et al. (2006) developed a multiplex PCR based on the major capsid protein gene using primer sets that could distinguish genotypes II and III and III and IV LCDV isolates in a mixed genotype sample.

Epizootiology Lymphocystis disease has been described in many species of teleosts from marine and freshwater environments (Anders 1989). The phylogenetically primitive bony fish families are infected as well as the more evolved ones. The families Centrarchidae, Percidae, Sciaenidae and Pleuronectidae are most commonly infected. Most records come from the North Sea and Baltic Sea fisheries in European waters, but Southeast Asia is also reported. In the north-east Atlantic, only Pleuronectidae species are often infected. In the coastal waters around the British Isles there are records of localities with high prevalence of Lymphocystis at Rye Bay plaice in the Irish Sea, at plaice off the coast of Cumbria and in the River Ythan Estuary on the north-east coast of Scotland.

In Asia, in 2011, lymphocytic diseases in Japanese clod cultivated in Japan (Tanaka et al. 1984), China (Sun et al. 2000) and Korea (Hossein et al. 2008) were reported frequently and are problematic as a harmful infection in this area valuable trades. In Korea, the lymphocytic disease is observed not only in farmed scorpion fish (Kitamura et al. 2006), but also in ornamental fish species such as stained glass fish, golden gourami and pearl gourami (Hossain et al. 2008). From a global point of view, in recent years lymphocystosis has proved to be an economic marketing problem not only in the countries of the Asia-Pacific region, but also on the Mediterranean coast of Spain, especially for the farmed sea bream.

control

Virus release occurs when lymphocysts rupture, and transmission is thought to occur by skin abrasion due to parasite or filament damage, or by mating behavior. There is no practical cure for the condition. Given that lymphocysts will eventually be shed and there is an immune

response to the virus, it is best to isolate affected fish from farmed fish as early as possible to avoid cross-infection and allow the lesion to heal. More recently, the conditions for stable incorporation of plasmid DNA into alginate microspheres with survival in the fish gut have been reported in Japanese plaice (Tian et al. 2008). In addition, fish orally vaccinated with pDNA-loaded alginate microbeads, in contrast to control fish vaccinated with naked pDNA, developed humoral antibodies up to 14 weeks post-vaccination. This type of experiment bodes well for vaccination therapy, and the next step is experimental oral vaccination against live lymphocytic virus infection.

White Sturgeon Iridovirus: Virus not assigned to any genus

This white sturgeon iridovirus disease (WSIVD), which has affinities for lymphocystosis, is nevertheless described separately due to certain differences. The development of freshwater sturgeon farming in several Pacific states of the United States has led to the emergence of several new viral diseases. WSIVD, which affects the seed coat and gills of juveniles and fry, is the most economically serious of these diseases today (Hedrick et al. 1990a). There is evidence that disease is stress-induced when virus carriers are reared in high densities

7.16 IRIDOVIRUS CAUSES DEADLY SYSTEMIC DISEASES

Genus Ranavirus

The genus Ranavirus includes teleost fish and amphibian viruses, but only teleost fish viruses are discussed in detail in this chapter. The study by Essbauer and Ahne (2001) comprehensively covered ranaviruses of all lower vertebrates. The eighth ICTV report (Chinchar et al. 2005) lists six groups of species: Ambystoma tigrinum virus (ATV), Bohle iridovirus (BIV), Epizootic Hematopoetic Necrosis Virus (EHNV), European Catfish (ECV), Frog Virus 3 (FV - 3).) and Santee-Cooper ranavirus (SCRV). ATV and FV-3 contain almost exclusively amphibian hosts. While BIV was isolated from the ornate ditch frog (Lymnodynastes ornatus) (in Bohle, Queensland, Australia), it was shown to be specific to a species of farmed fish, the barramundi (Lates calcarifer) (Moody & Owens, 2004).). EHNV, ECV and SCRV are the groups comprising bony fish isolates. Whittington et al. (2009) studied the Ranaviridae of fish and their epidemiology.

7.17 ADENOVIRIDAE

Adenoviruses are common in humans and primates, where they are primarily associated with respiratory and gastrointestinal diseases. They are also common in domestic animals, including birds. Many are isolated from asymptomatic infections. Some are oncogenic and can transform cells in vitro. Adenovirus has an unenveloped icosahedral virion with a diameter of 70-90 nm and a particle mass (Mr) of 170-185 x 10 6. There are 252 capsomeres arranged in pentagons, with the apex capsomeres bearing a peripheral fiber with a terminal button. The genome consists of linear double-stranded DNA of 20 to 30 x 10 6 M r. There are at least nine structural proteins in the virion. Viral infectivity is ether resistant, acid stable and inactivated at 56°C for 10 minutes.

7.18 HERPESVIRIDS

Herpesviruses are complex viruses that cause a variety of diseases in humans and animals, including fish. Herpesviruses have some interesting properties; some cause cancer and evidence has been presented for some tumor-causing fish herpesviruses. Human diseases include cold sores, chickenpox, and shingles, in which the virus can remain dormant in the host for long periods, only becoming active under certain conditions such as stress, season, and endocrine cycles, all of which may be interrelated. Some fish herpesviruses appear to produce similar effects.

The herpes virion is an enveloped spherical particle with a diameter of 120-200 nm, approximately Mr 10 × 10 8 and a complex architecture. There are four different morphological units; in the center is a protein coil around which linear double-stranded DNA, M r 70 - 150 × 10 6, is wound. Surrounding the nucleus is a nucleocapsid with icosahedral symmetry, consisting of 162 capsomeres, each of which consists of several proteins. Outside the nucleocapsid is an amorphous layer called the integument, a fibrous-looking structure specific to herpesviruses. The integument is surrounded by an envelope comprising a lipid bilayer containing at least six glycoproteins that protrude as spikes. DNA codes for many different proteins, more than 20 of which are structural proteins. Infection occurs through the attachment of virions to specific cell receptors, and after fusion of the cytoplasmic membrane with the viral envelope, the nucleocapsid is released into the cell. The nucleocapsid is transported to the host nucleus where the viral DNA is uncoated. Components of the virus particle inhibit macromolecular synthesis by the host. Viral nucleocapsids are assembled in the nucleus, and

acquisition of the viral envelope occurs via a process of budding through the inner membrane of the nucleus. Mature virions are then released to the outside of the cell through the endoplasmic reticulum. Useful criteria for confirming that a virus belongs to the herpes family are described by Buchanan and Madeley (1978) and further listed as turbot herpesvirus, herpesvirus psetti.

7.19 RNA VIRUSES

reovirides

The name REO comes from "respiratory - enteric - orphan" and refers to the tissues most affected by the disease (i.e. the respiratory and gastrointestinal tract), while in the case of "orphan" is the isolation of a virus with no identifiable disease caused. Reoviridae have been isolated from mammals, birds, fish, molluscs, crustaceans, insects and plants. Fish reovirus isolates have been assigned to the genus Aquareovirus since 1995 (Lupiani et al. 1995). Older articles sometimes referred to the isolates as members of the rotavirus genus Reoviridae. The reovirus has an unenveloped virion with two concentric icosahedral capsids and is 60-80 nm in diameter, and M r estimates range from 65-160 x 10 6. The genome consists of 10-12 double-stranded RNA segments, M r 12-20 x 10 6. Virions contain 10-12 structural polypeptides, some glycosylated, and have transcriptase activity.

7.20 AQUAREOVIRIDAE

There have been more than 30 aquareovirus isolates from freshwater and marine fish (reviewed by Lupiani et al. 1995). Many have not been associated with clinical pathology, consistent with the orphan description of reoviruses of other genera (ie, not associated with known disease). The Aquareovirus genus has been extensively described by Samal et al. (2005), and the evolutionary origin and phylogeny of aquareoviruses compared to orthoreoviruses has been reported and debated.

7.21 PEAR VIRUS

Birnaviruses are isometric, envelopeless, spherical viruses containing two segments of double-stranded RNA. Hence the name: bi stands for the bisegmentation of the viral genome and its double-stranded character and arn for the type of nucleic acid. The family includes three main genera classified by the International Committee on Taxonomy of Viruses (Delmas et al. 2005):

the genus Aquabirnavirus (with the type species Infectious Pancreatic Necrosis Virus, IPNV) many fish species, the genus Avibirnavirus (type species: Infectious Chicken Bursal Disease Virus, IBDV) and the genus Entomobirnavirus (type species, Drosophila X, fruit fly virus, Drosophila sp.) The IPN virus was the first fish virus isolated (Wolf et al. 1960) and is one of the best studied fish viruses in Molecular Biology, Recognition and Animal Health. Birnaviruses are associated with a variety of host pathologies based on a combination of host, viral and environmental factors affecting birnavirus pathology and/or virus persistence along with outbreaks of all infectious diseases (Snieszko 1974).

7.22 PICORNAVIRIDES

The family name is derived from pico (small) and RNA. Economically important members of this family include foot-and-mouth disease, swine vesicular disease, rhinoviruses that cause respiratory infections (colds) in humans, cattle, and horses, and enteroviruses that replicate in the digestive tract and oropharynx, including poliovirus. Members of this family are very small, non-enveloped, positive-sense, single-stranded RNA viruses, 20-30 nm in diameter and with icosahedral symmetry. The viral capsid consists of 60 structural units, each containing one of the four main polypeptides of the virion. The M r of the virion is 6-9 x 10 and the M r of the RNA is about 2.5 x 10 6 or about 30% by weight. Infectivity survives ether and pH 3.

7.23 NODAVIRIDS

Until the discovery of nodavirus infections of fish, called betanodavirus, genus Betanodavirus, (Schneemann et al. 2005), members of this family consisted exclusively of insect viruses, the alphanodaviruses. Nodaviruses are small, envelopeless, icosahedral viruses approximately 25-35 nm in diameter. The genome has a positive-sense RNA consisting of two single-stranded molecules of Mr 1.17 and 0.48x10 6.

7.23.1 Fish nodavirus

Diseases caused by Noda and Noda-like viruses in fish have been given different names. The disease was first described by Bellance and Gallet de Saint-Aurin (1988) in farmed sea bass and later named viral nerve necrosis (VNN). Other names are Striped Bonito Nerve Necrosis (SJNN) and Viral Encephalopathy and Retinopathy (VER), which are commonly used in the literature. The first two reviews of aquatic nodavirus disease are from Munday and Nakai (1997) and Castric (1997) (Table 6.9). Munday et al. (2002) studied betanodavirus infections

of teleost fish, listing 32 VNN host species by order and fish family. Five orders are represented: Anguilliformes, 1 host species, Gadiformes, 1, Perciformes, 24, Pleuronectiformes, 5, and Tetraodontiformes, 1. In the order Perciformes, groupers (family Serranidae) have the largest number of host fish with 9. Since the time of this review, new host species have been discovered (e.g. haddock) (Gagné et al. 2004; Murray et al. 2010)., and the approximate total is 40 worldwide.

7.24 TOGAVIRIDE

The name derives from the Latin toga, a cloak, a reference to the virus envelope. Togaviruses cause encephalitis in horses, swine fever in pigs, diarrhea in cattle and humans. The rubella virus causes developmental disorders when infection occurs early in pregnancy. Some are carried by arthropods. The virion is spherical, 50-70 nm in diameter, and comprises an envelope with surface protrusions composed of 2-3 polypeptides, usually glycosylated. The nucleocapsid has a protein core and a single strand of positive RNA, M r 4 × 10 6. Maturation occurs by budding of icosahedrally symmetrical nucleocapsids through cytoplasmic membranes. Togaviruses are inactivated by lipid solvents and ionic and non-ionic detergents.

7.24.1 Fish togaviruses

Atlantic salmon pancreatic disease (PD) and rainbow trout sleeping sickness (SD) are caused by related isolates of salmonid alphavirus (SAV), a species in the genus Alphavirus, family Togaviridae. Salmonid pancreatic disease virus and sleeping sickness virus were virus names used in the literature prior to 2005, and both appear in the eighth ICTV classification list (Weaver et al. 2005). SAV was reviewed by McLoughlin and Graham (2007). Erythrocyte inclusion body syndrome (EIBS) is an infection associated with a Toga-like virus that has not been cultured.

7.25 PARAMYXOVIRIDAE

Members of this family cause a variety of diseases in higher animals, including distemper and Newcastle disease, but few are reported in fish. Virions have a morphology similar to Orthomyxoviridae but are larger, around 150–200 nm and sometimes larger. The envelope consists of a lipid bilayer containing a matrix protein. The bilayer is spiked and, as in Orthomyxoviridae, consists of two proteins important for attachment and penetration of host

cells. The genetic material is single-stranded, unsegmented, negative-sense RNA. RNA is surrounded by a nucleoprotein to give a nucleocapsid.

7.25.1 Fish Paramyxovirus

The first paramyxovirus disease detected was that of Winton et al. (1985) who isolated the virus from healthy adult chinook salmon stocks. The virus was not pathogenic to chinook salmon but, like paramyxoviruses of higher animals, has been reported to cause hemagglutination of fish and other animal red blood cells in vitro (Lannan et al. 1989). The virus is restricted to growth in CHSE-214 cells and some other syncytia-producing salmonid cell lines. It grows slowly and is temperature labile with an optimum of 18 °C. Coated particles of 125-250 nm containing nucleocapsids were observed by TEM (Figure 6.47). More recently, genetic analysis of Pacific salmon paramyxovirus (PSPV) revealed two independent, co-circulating lineages (Batts et al. 2008), the B sublineage (Yaquina River, Oregon, 1982) and the A sublineage (Trask River, Oregon). 1983).

Miyazaki et al. (1989) reported that a Paramyxo-like virus caused an outbreak characterized by epidermal necrosis in black seabream larvae. The disease appeared in fry from 25 to 30 days of age and resulted in 100% mortality. The disease could be transmitted with almost 100% mortality by immersing fish in an ultrafiltrate of naturally infected fish 4 to 6 days after challenge. The virus has not yet been cultured. The pathology is characterized by necrosis of epithelial cells of the epidermis of the fins and body surface, gills, intestinal and oral mucosa. Paramyxo-like virions of 300–370 nm were observed in the cytoplasm of infected cells by TEM. Atlantic salmon gill paramyxovirus (ASFV) is a novel paramyxovirus (Kvellestad et al. 2003) that contributes to the etiology of proliferative gill inflammation in Atlantic salmon reared in seawater (Kvellestad et al. 2005). Cytopathic effects were observed in Rtgill-W1 cells inoculated with gill tissue material 9 weeks post-inoculation. CPE was characterized by rounding and shrinking of cells with visible cytoplasmic inclusions in stained infected cells. Later, syncytia formed, which are typical for paramyxoviruses. The negatively stained virions were spherical and partially pleomorphic, 150-300 nm in diameter, and the virus contained both hemagglutination and receptor-destroying enzyme (RDE) activities, the RDE being due to a neuraminidase. Five major structural polypeptides and a floating virion density of 1.18-1.19 g/mL in CsCl gradients have been reported. Recently, Falk et al. (2008) reported the molecular characterization of ASFV. In the family tree of Paramyxovirus genera, ASFV most

closely corresponds to the genus Respirovirus, but differs from it and may represent a new genus.

7.26 ORTHOMYXOVIRIDAE

Orthomyxoviruses include the influenza viruses, which are best known to cause disease in humans but are also common in other mammals and birds. Virions are pleomorphic, roughly spherical or filamentous, 80-120 nm in diameter or cross-section, M r 200-400 x 10 6. There's an outer case that's riddled with protrusions. The envelope consists of a host lipid bilayer and is lined with the matrix protein and traversed by several hundred needles. Each spike consists of a fixed ratio of spike proteins. Spike proteins play an important role in host cell attachment and release. The genomic material consists of negative-sense single-stranded RNA in the range Mr 4.6 - 6.4 x 10 6. RNA is made up of eight separate segments held together by a nucleoprotein. The segments encode two envelope, matrix and nucleoprotein spike glycoproteins, three viral polymerases and 1-3 nonstructural proteins (found in infected cells but not in virions). Plasma membrane virus buds.

7.27 RHABDOVIRIDS

The name rhabdo means "stem" and refers to the bullet-like shape of the virus particle. The best-known rhabdovirus is the rabies virus, which causes severe neurological diseases in warm-blooded animals, including humans. It is primarily a zoonosis of wild animals, mainly carnivores, but domestic animals are also affected. Another widely studied member of the Rhabdoviridae is the vesicular stomatitis virus, which causes disease in cattle, swine, horses, and sometimes humans. Other rhabdoviruses, such as Potato Yellow Dwarf Virus, infect both plants and insects, causing significant problems in agriculture. The virions are characterized by their shape and by the helical nucleocapsid encased in a lipid envelope bearing surface protrusions. The virions are 120–380 × 60–90 nm in diameter, giving the hollow core a diameter of about 50–55 nm. Rhabdoviruses contain unsegmented, single-stranded, negative-sense RNA. The linear genome encodes five proteins N, the nucleocapsid protein, two structural proteins, G the glycoprotein and L, the RNA polymerase. Virions are sensitive to lipid solvents, detergents and proteolytic enzymes and are rapidly inactivated at 56 °C and pH 3. Lipids make up 15–25% of the virion, carbohydrates about 3%, RNA about 1%, and proteins the rest.

7.28 FISH RHABDOVIRUS

Members of this large group, isolated from bony fish, are implicated in some of the most serious economic diseases in aquaculture. Of the genera currently recognized in the Rhabdoviridae (Tordo et al. 2005), all fish rhabdoviruses are now placed in two such genera, Vesiculovirus and Novirhabdovirus. Infectious hematopoietic necrosis virus (IHN), viral hemorrhagic septicemia virus (VHSV), and hirame rhabdovirus (HIRRV) contain a non-virion gene (NV) (Kurath & Leong 1985) that is not present in other rhabdoviruses, as well as the common conserved gene junction sequences and similarities in their nucleotide sequences of the N, M1, M2, G and NV genes (Morzunov et al. 1995; Bjorkland et al. 1996; Kurath et al. 1997; Nishizawa et al. 1997b). This evidence led to the grouping of these viruses into the genus Novirhabdovirus (non-virion) as reported by Nishizawa et al. suggested. (1997a). Their distinction is most easily seen in dendrograms generated by CLUSTAL analyses, in which the deduced amino acid sequences of the N, M1 and M2 genes of several members of this new genus were compared to prototype members of lyssavirus and vesiculovirus.

7.28.1 Fish Retroviruses and Related Diseases

In a review of fish retroviruses, Bowser and Casey (1993) listed 13 neoplastic or proliferative lesions in fish with retroviral or suspected retroviral etiology. The retrovirus has also been isolated from cell lines derived from tumorigenic material as well as from normal tissue (see below). Repetitive DNA sequences similar to the long terminal repeats of retroviruses have been found in salmonids (Moir & Dixon 1988), and Stuart et al. (1992) describe the discovery of DNA sequences similar to the retrovirus pol gene in several salmonids.

CHAPTER 8

MISCELLANEOUS NON-INFECTIOUS DISEASES

According to Parker, non-infectious diseases are not caused by pathogens, which are usually related to environmental factors, inadequate nutrition, or genetic abnormalities. Successful fish health management includes preventing disease, reducing the incidence of infectious disease, and reducing disease severity. Avoiding contact between susceptible fish and a pathogen should be a primary goal in preventing an outbreak of an infectious disease. According to Winton's report, the three main actions to achieve the goal are using a pathogen-free water supply, using certified pathogen-free stock, and observing strict hygiene practices, as implementing these actions reduces the exposure of fish to pathogens. However, it is virtually impossible to define all pathogens in the aquatic environment and completely prevent host exposure to pathogens. Certain factors, such as overcrowding, increase the susceptibility of fish to infection and the transmission of pathogens. Therefore, many pathogens that do not cause disease in wild fish can cause outbreaks with high mortality rates in high-density fish production systems. Similarly, fish diseases are a significant source of financial losses for aquaculture operations. Production costs are increased by outbreaks of fish disease due to lost investment in dead fish, treatment costs, and reduced growth during recovery. In the wild, people are less aware of the problems associated with fish disease, as predators quickly wipe diseased animals out of the population. Also, fish are much less numerous in natural systems than in captivity. Pathogens can be of minimal concern under natural conditions, but can cause significant problems when animals are overcrowded and stressed under housing conditions (Ruth). Disease is rarely a simple link between a pathogen and a fish farmer, and therefore management practices aimed at limiting stress are probably the most effective at preventing outbreaks.

There are various fish diseases caused by bacteria, virus, parasite, fungus, environmental problem, malnutrition, etc. negatively affecting aquaculture. These diseases are divided into contagious and non-contagious diseases. Infectious diseases are diseases caused by pathogenic organisms present in the environment. In contrast, non-infectious diseases are diseases caused by environmental problems, nutritional deficiencies, or genetic conditions; and they are not contagious and usually cannot be cured by medication (Ruth). However, both infectious and non-infectious diseases are causes of fish disease and some impacts, including direct deaths, productivity losses due to reduced growth, fertility, product quality and social factors, and the

cost of control measures. Therefore, assessment of non-communicable diseases is important to develop fish biosecurity management in aquaculture for disease prevention and control methods.

8.1 NON-INFECTIOUS FISH DISEASES:

Non-communicable diseases, also called non-genetic or non-communicable diseases, are diseases that are not caused by pathogens and cannot be transmitted from one host to another. They are emerging and re-emerging diseases in aquaculture and are characterized by lesions, deformities, anorexia, anemia, weight loss, asphyxiation and death when severe. This makes the fish unsaleable as it is considered poor quality by processors (dung). Non-infectious diseases can be broadly classified as environmental, nutritional, or genetic (Ruth). In general, fish diseases related to the physicochemical properties of the water are non-contagious environmental diseases. Non-contagious diseases are caused by adverse environmental conditions, nutritional disorders or genetic defects and lead to mass mortality or sudden death, they are not contagious. Diseases related to physical factors are mainly due to handling, transportation, high stocking density and predation, while secondary factors such as bacterial, viral or parasitic infections can easily occur once injuries have been introduced. Diagnosis of non-infectious environmental diseases can be made by examining fish for external/internal signs of disease; histopathological/histochemical analyses; hematological analysis to assess the cellular composition of blood in response to environmental stress; Analysis of the physico-chemical properties of the rearing waters; and evaluating cultural operations and management practices. Host effects can include death from blood embolism and tissue emphysema; edema and degeneration of gill lamellae; swelling of the cornea; sudden mass extinction. Therefore, prevention and control methods include monitoring dissolved oxygen (DO), preventing algal blooms, maintaining efficient operation of water pipes and pumps, adequate water exchange, etc. (ErazoPagador).

Environmental Diseases of Fish: Environmental diseases are the most important in commercial aquaculture. Environmental diseases include low dissolved oxygen, high ammonia, high nitrite, or natural or man-made toxins in the aquatic environment. Proper water quality management techniques allow growers to prevent most environmental diseases.

Nutritional Diseases in Fish: Nutritional diseases can be very difficult to diagnose. A classic example of a catfish feeding disease is Broken Back Disease caused by vitamin C deficiency.

A deficiency in dietary vitamin C contributes to poor bone development, leading to spinal deformities. Another important nutritional disease of catfish is "absence of blood disease" which can be related to folic acid deficiency and affected fish become anemic and may die. The condition seems to disappear when the deficient food is discarded and a new food is provided (Ruth). A non-pathogenic disease such as an unbalanced diet causes fatty liver disease (Fig.1)

Figure 1: The non-pathogenic disease of an unbalanced diet causes fatty liver disease (AFCD, 2008).

Genetic Defects: Genetic defects include conformational peculiarities such as the absence of a tail or the presence of an extra tail. Most of them are of minimal importance; However, it is important to bring unrelated fish to use as breeding stock every few years to minimize inbreeding (Ruth).

8.2 PATHOLOGY OF NON-INFECTIOUS DISEASES:

Similar to the pathology of infectious diseases, non-infectious diseases have their own symptoms and diagnoses, modes of transmission, prevention and control measures, and treatments.

Fish Disease Symptoms and Diagnosis: Accurate diagnosis and quick response will stop the spread of disease to other fish and minimize losses. Symptom and Diagnosis of Fish Diseases: Darkening of the skin, sluggishness, surface swimming, shortness of breath, stopping eating, abdominal swelling, enlarged liver and spleen, focal blood clots, eye swelling, gill discoloration, etc. are suspected of infection. Based on the OIE Manual for the Diagnosis of Aquatic Animal Diseases (2000a), behavioral changes are not specific to pathogens but may include lethargy, aggregation in quiet areas of the pond with flares, periodic erratic swimming, and loss of balance. Changes in appearance include darkening of the body, particularly in the yolk sac brood stage (90–100% mortality). The abdomen may be bloated (dropsy) due to accumulation of fluid in the body cavity (AGDAFF, 2007). Infected fish showed darker skin,

abdomen may be bloated, bleeding at base of fins, operculum and around eye, impaired ability to swim, bleeding at base of fins, white discharge from anus (Fig 2)

Figure 2: Infected fish with white discharge from the anus, impaired buoyancy, darkening of the skin, pale gills

8.3 TRANSMISSION OF FISH DISEASES:

The disease is primarily transmitted from fish to fish by direct contact or through water via feces, urine, genital secretions, or external mucus. The manifestation of the disease depends on the size and age of the fish, species and strain, and environmental conditions, including water temperature. Horizontal transmission from fish to fish through water is common in nature and on aquaculture farms, mainly in juvenile and juvenile fish and also older fish. Transmission is usually through water, as all the secretions and excrements of infected fish are present. Blood-sucking parasites and fish-eating birds can be introduced to new areas (AGDAFF, 2007). The route of transmission is most commonly spread during excretion with feces, urine, spawning fluids, mucous secretions, contaminated equipment, infected fish eggs, and blood-sucking parasites (e.g., leeches, Argulus spp.). In general, the pathogens are transmitted through various routes such as feces, urine, sexual fluids, external mucus, and through direct contact or close contact with the surrounding contaminated water, and infected fish spread the disease in fish.healthy fish, fish that can survive carriers.

Fish Disease Prevention and Control: Prevention is the cornerstone of any health protection program and can be as difficult and complex as actually controlling existing diseases. Key elements of disease prevention include knowledge of water quality control methods, pathogen transmission, reliable detection of disease vectors, development of effective methods to limit pathogen entry, ability to create conducive environments for healthy fish, and movement

preventing infected individuals fishing between watersheds, reducing bird activity in aquaculture, biological and chemical methods, reducing physiological stressors (Ayalew Assefa and Fufa Abunna). Good preventive medical practices include quarantine, routine surveillance, vaccination, use of immunostimulants, probiotics, and diagnostics for disease management (Roy and Claire). Many other important elements of fish health management need to be considered prior to regulation such as: Rapid advances have been made in research on the immune system, such as facilities, water supply, environmental manipulation, diet and feeding, genetic resistance diseases, vaccination, etc. fish responses and in the development of immunization procedures (Plant and LaPatra). Hygiene and disinfection measures to reduce the risk of recontamination (Francis). Protecting aquaculture facilities in these circumstances requires adequate disinfection of equipment and inlet water (Whittington). Control measures include destocking, cleaning, and disinfecting with appropriate treatments that contribute to the eradication of an aquaculture facility (Bryan). The further development of vaccination is one of the most important approaches to the prevention and control of fish diseases (Dadar). Vaccination is widespread in almost all food-producing animals. In aquaculture, it reduces the use of antibiotics to protect fish from disease and avoids the risk of drug resistance (Plant and LaPatra). A few important considerations before vaccinating fish include the species of fish to be vaccinated, the status of the fish's immune system, the production cycle and life history of the aquaculture system, what diseases need to be controlled in aquaculture, when these diseases appear (seasonal distribution of diseases in the aquarium), agricultural engineering (handling and mechanization), environment (temperature and salinity), stressors, nutrition and profitability (Adams).

Treatment of fish diseases: According to the AFCD report (2008), medicated bathing is an important treatment of fish diseases. Preventive measures are still recommended due to the lack of an established treatment. However, some of the effective control measures are vaccinations, antibiotics, and bathing (Ruth). Effective disease control involves a thorough fish health management program that eliminates infected stock, prevents reinfection, reduces stress and maintains optimal production conditions. When the fish are provided with a good environment and adequate nutrition, the risk of infection from pathogens is greatly reduced. But chemotherapy, antibiotics, baths, etc. are part of the treatment of diseases. Chemotherapy is the use of drugs and chemicals to treat infectious diseases and pathogens without significant adverse effects on the host fish. Antibiotics are very useful additions to a fish health manager's tool kit, but they are just tools and not 'quick fixes'. The immersion treatment consists of a short

immersion bath lasting from a few seconds to 5 minutes, depending on the chemical used and its concentration, and is often used on broodstock. Although effective, they can be very stressful. After treatment, fish should be rinsed with clean water before returning to the storage facility to prevent transfer of chemicals into the tank. Considerable attention to sources of stress such as poor water quality, diet, genetics, handling, or transportation should be removed or reduced. Contacting a fish health specialist early on in the event of a disease outbreak will help identify stressors and the rate of bacterial infection, thereby reducing losses.

There are many fish diseases and anomalies that do not easily fit into any of the broad categories in the previous sections. There are a number of non-infectious diseases associated with physical or chemical changes in water, diseases directly associated with genetic aberrations, and diseases of known origin that do not belong to any particular category, and there are several known diseases whose etiology is still opaque. Because they have little in common with each other or other types of diseases, they are discussed here under Other Non-Infectious Diseases.

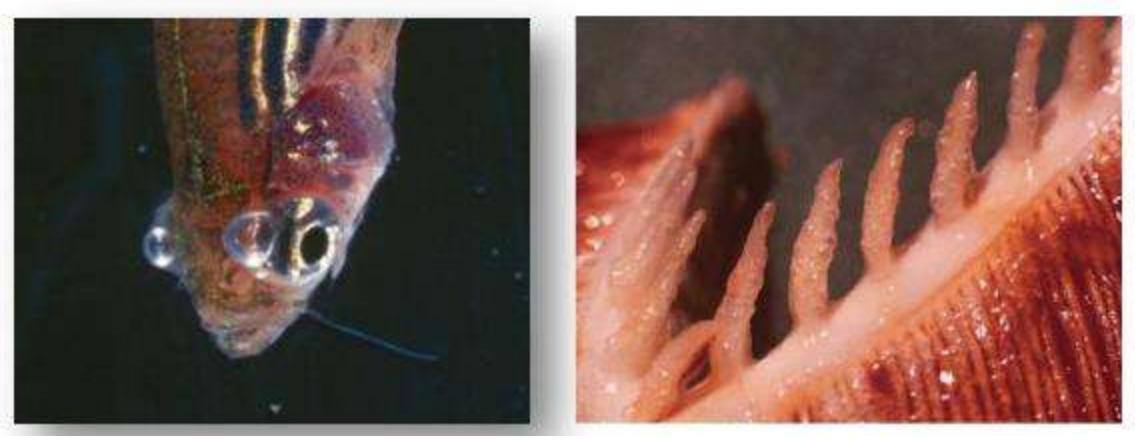
8.4 GAS BUBBLE DISEASE

One of the most important farmed fish diseases is the disease commonly known as gas bubble disease. First described in aquarium fish by Gorham in 1898, it was originally thought to be a problem primarily for fish downstream of a powered hydroelectric power station. It has now been observed clinically in a variety of crops and in a number of different circumstances (Marsh & Gorham 1904; Alikunhi et al. 1951; Harvey & Cooper 1962; Rucker 1975; Saeed & Al-Thobaiti 1997). This, coupled with the recognition that it reflects hyperbaric problems in humans (Speare 1998), has led to widespread interest in its pathophysiology. Outbreaks in wild fish are less common but have occasionally been reported in freshwater and marine fish.

Although originally associated with the effects of dissolved nitrogen, it is now recognized that supersaturation is a function of the total pressure of the dissolved gas and that it is this pressure and not the partial pressure of nitrogen that is indicative of potential bubble disease (Weitkamp & Katz 1980). In aquariums and hatcheries, the condition can be caused by leaks in pump or valve systems, or by sudden temperature gradients. It has been associated with changes in height of fish transported by air (Hauck 1986). This remains a concern given the proliferation of air transport of tropical aquarium fish and the widespread use of helicopters to transport live salmonids. In nature, vigorous algal blooms that produce locally excessive concentrations of

dissolved gases have been involved, but the physiological mechanism of this syndrome, so similar to the "diver's" syndrome in humans, is still poorly understood, despite its importance. in many types of intensive fish farming. However, fish farming systems have the greatest potential for sudden and severe outbreaks. As production levels have increased with the advent of water recirculation and the simultaneous use of air and oxygen injection, it has been shown that even oxygen injection alone can induce gas bubble disease due to oxygen supersaturation.

The degree of supersaturation is the most important factor in terms of both the clinical picture and the end result, but the duration of exposure and subsequent treatment also influence survival. Fish often die from gas bubble disease without overt clinical signs, and the mortality rate varies with the age and species of fish affected. In fish larvae, where this is particularly problematic, the gas bubbles in the subcutis and yolk sac are more visible (Fig. 11.1), although in flatfish larvae the edges of the body fins appear to be particularly susceptible to gas emboli. In older fish, bubbles are most commonly seen in all chambers of the eye, skin, gills, and mouth, but internal gas accumulation is also found in the swim bladder and visceral peritoneum at autopsy.



The sacs in the eyes are usually blind and therefore darker in color. Often the end result of damage caused by expanding gas bubbles in the eye is blindness and eventually consumption.

The histopathology of the condition was described in a small number of Chinook salmon by Pauley and Nakatani (1967) and it is on this rather fragmentary study and the review by Speare (1998) that the present description is based. based. The main consistent histological feature observed is edema of the secondary lamellae of the gills with concomitant degeneration of the overlying respiratory epithelium. Edsall and Smith (1991) showed that intravascular gas embolism leads to occlusion of large branchial vessels and that this is a major cause of acute

mortality. Other lesions, including edema and bullous rupture of the oral and intestinal mucosa and vacuolar degeneration of the renal tubular epithelium, have been described as part of a general syndrome also involving liver and muscle changes.

It's unclear how the disease will affect the future performance of fish recovering from an outbreak. Pauley and Nakatani (1967) were probably correct in emphasizing the importance of variables such as size and species of fish involved, degree and duration of supersaturation, and water temperature in determining the prognosis of these fish.

8.5 DISEASES AT LOW TEMPERATURE

The connection between low water temperatures and flavobacterial diseases has already been discussed (Chapter 8). However, in cultured salmonids and pleuronectids, hyperplastic epidermal disease can occur without any evidence of flavobacteria involvement, except as a peri- or post-mortem invader. A hyperplastic fin and tail condition associated with high mortality rates has been identified in plaice at low water temperatures in Scotland (Roberts, unpublished) and RE Wolke (personal communication) described a similar condition in coho salmon farming in the eastern United States. Conditions.

Johansson (1968) described a proliferative condition of the gills of 2-year-old Atlantic salmon in freshwater fish at water temperatures below 3°C. Extreme sensitivity to ammonia at low temperatures and to pantothenic acid deficiency with loss of appetite were suggested as possible causes but not confirmed. The disease appears to develop in two stages: the first, with low mortality and hyperplasia of the gill lamellae at the base, is followed by 20-30% mortality after 2-6 weeks, during which microscopic examination of affected fish shows a fusion of secondary lamellae.

A serious problem associated with low water temperatures is 'water belly', a profuse accumulation of fluid in the abdomen which is not uncommon in saltwater salmon and particularly rainbow trout at higher latitudes. The stomach is greatly enlarged and filled with seawater, the extent is so great that the stomach and its contents can account for up to 40% of the total body weight.

The main causative factor is osmoregulatory stress associated with low temperature and high salinity. Trout, which are less effective at dealing with the osmotic problems of saltwater existence, are more commonly affected, but this can be a problem in a small number of salmon,

particularly when switched from a lower temperature diet (Figure 11.3).. This condition appears to differ from that reported by Lumsden et al. (2010) relating to nutrition.

8.6 WATERBORN IRRITANTS

Each species of fish has an optimal range of water pH values, and the importance of these and the factors that affect them were discussed earlier in Chapter 1. However, it is worth remembering their importance and pointing out that many of the factors that cause a rapid change in pH are also harmful to the gills for other reasons. Particular irritants such as cement dust or slurry and ammonia cause gill irritation damage that may not be immediately clinically apparent. In fact, the gill response to such irritation is usually proliferative and does not develop immediately, and since at low temperatures, when the solubility of oxygen in water is high, the fish usually has a more than adequate respiratory reserve. Clinical effects only become apparent at high temperatures with low oxygen tensions.

Daoust and Ferguson (1985) described a unique form of proliferative gill disease in rainbow trout thought to be of environmental or parasitic origin, which they termed nodular gill disease. In contrast to the more common forms of proliferative gill disease associated with parasitism or bacterial infection, the lesions are nodular rather than diffuse and usually connect multiple lamellae. They consist mainly of squamous epithelial cells forming hyperplastic masses. Dramatic gill hyperplasia was a feature of some fish species reared in recirculating aquaculture systems where total ammonia levels exceeded 30 mg/L (Rodger unpublished). However, it is unclear whether this pathology is the direct result of high levels of ammonia or a reflection of other adverse environmental parameters.

8.7 BLUE SACK, WHITE spot and DANGEROUS SACK DISEASE OF LARVAE

Losses of salmonid larvae, which are due to various components of the dissolved water, including metal ions, ammonia, lack of gravel in smooth-surfaced brood pans, and silt, belong to this group of larval conditions associated with swelling, deformation, or discoloration of the yolk sac.. Color can vary from light blue to gray and whitish spots can appear in the yolk or on the surface of the sac where they are due to focal spongiosis and hyperplasia of the pouch epithelium most often associated with excessive amounts of antifungal treatment chemicals.. The yolk sac can also be pinched at the top to create a dumbbell shape. Losses can be very high and although in most cases the cause is imprecisely defined, their occurrence usually indicates that the hatchery water supply is unsuitable.

8.8 COLOR ANOMALIES

Many color anomalies in fish are genetic and often the basis for ornamental varieties or strains. These are discussed in this chapter. However, pseudoalbinism, a very common trait of farmed flatfish, appears to be a breeding-related developmental anomaly. Partially pigmented or inversely pigmented animals are present in significant numbers in almost all hatcheries. The exact etiology is not known. The lesion is believed to develop at an early age and is associated either with the relatively very high level of lighting used in these hatcheries to maintain live feed levels, during the critical early stages of feeding, or with the lack of certain essential nutrients in will be connected as soon as possible. Food. Once established, the deviant pattern persists throughout life.

8.9 PHYSICAL DEFORMATIONS

Physical deformities can have a variety of causes. Congenital malformations, which are not uncommon in inbred populations, may include malformations of the jaw or skull, abnormalities of the gills or spine, or more commonly extra-pectoral fins or shortening of the operculars (see below). Other causes range from incubating eggs or larvae at temperatures that are too high, low mineral levels in the larval diet, teratogens in food or the environment, and the use of hormonal manipulations.

Feeding or rearing practices particularly affect spinal development, resulting in scoliosis, lordosis, or occasionally a deformity of the jaw, mandible, or maxilla. Lesions related to genetic, incubation temperature, or hormonal effects are evident early in hatchery development, while breeding or dietary lesions, which will be very prevalent in affected flocks, may occur at any developmental stage.

In addition to the diet-related skeletal abnormalities described in this chapter and in Chapters 3 and 10, a variety of other disorders can occur in certain batches of fish due to genetic effects. The condition of cross-platy swordtail malignant melanoma has already been described in chapter 5 on neoplasia. It is the only directly fully demonstrated gene-mediated condition of teleosts to date, but there are several other abnormal developmental conditions thought to be genetic in origin. These include the high frequency of congenital anomalies of the Siamese twin type seen in some salmonid matings and a wide range of other malformations (Gemmill 1912) including protruding and lower jaws, abnormal fin development and shortened growth of the operculum.

8.10 Mortality syndrome in the first life

In the Baltic Sea and the Great Lakes of North America, a number of reproductive problems have been identified in wild and hatchery-reared egg and larval salmonids and clupeids. Each case is slightly different, but all share common characteristics and are collectively referred to as early mortality syndrome (EMS). In Swedish salmonids, the disease is associated with certain families of fish (Lundstrom et al. 1998) and a high percentage of the offspring of certain females, usually smaller eggs, die between 10 and 20 days after hatching. The male does not appear to play a role in transmission of the disease.

Although the affected larvae showed clinical signs indicative of nerve damage, no histological changes were found in the nerve tissue and organochlorine residue assays in parent fish and offspring were insignificant. Similar conditions are found in gadoids, pleuronectids and clupeids from the Baltic Sea (Norrgren et al. 1998) and this reflects the situation in the Great Lakes where mainly salmonids are involved. Thiamine deficiency appears to be a common trait of affected offspring, but is not thought to be primary, particularly in wild fish. Overall, this condition is seen as a new paradigm in environmental issues that is not the result of a single overt toxicity, but rather the result of a series of potentially sublethal attacks.

8.11 CYSTIC CONDITIONS

There are a variety of cystic conditions found in fish, but a genetic cause for them has yet to be proven. In most cases, a parasitic origin, particularly cestodes, is the most likely. Multiple cystic conditions are common in salmonids when a parasitic etiology cannot be inferred. Roberts and MacRitchie (1971) described such an eruption in a group of brown trout (Figure 11.8), and Roberts and Hastein (unpublished) examined a series of very large (about 2 cm in diameter) cystic structures (Figure 11.9) associated with coarse ones gas. In the latter case, a large number of farmed large Atlantic salmon were involved, and the numerous clusters of pisiform cysts, filled with a clear amber-colored fluid but showing no sign of parasites, were distributed throughout the visceral peritoneum and also in the retroperitoneal parenchymal viscera. Bruno and Ellis (1986) described similar lesions in several populations of farmed salmon, except that they contained adipose tissue in addition to fluid, which was replaced with fluid with increasing size. Although the external appearance of gastric dilatation is similar.

8.12 TRAUMATIC INJURIES

In the wild, traumatic injuries usually result from an attack by a predator and include stab wounds, bites, and scratches. These usually heal by granulation unless secondary infection is present (Fig. 11.11). In farmed fish, both the handling required during sorting or transfer and the skin trauma associated with some types of enclosures can cause serious external damage. Within wire cages and enclosures, there is considerable species variation in the degree of composure with which confinement is accepted, and some species or individuals may continually damage their skin, particularly the snout, in attempts to escape. This is particularly common with Atlantic salmon when they are reared in extremely dark conditions in fresh water and then transferred to room temperature - clear sea - or fresh water cages. They dive to the bottom of cages or well boats and are often severely traumatized. These lesions can be hemorrhagic and erode down to the skeletal level. Secondary infection with flavobacteria or vibrios can result in high mortality rates and bizarre deformities even if cured.

Deformation also occurs when fish larvae are even slightly damaged during handling. Such fractures or dislocations usually heal, but in a distorted manner. A similar spinal malformation has also been reported in planktonic larvae of marine species in years where unusual current reversals or buoyancy have occurred.

Excessive currents combined with draining reservoirs too quickly or excessive current speeds can cause pectoral nets to break in salmon as they approach the smolt. The lesion appears to grow as they spread their fins to avoid being washed backwards by the current. The fin mesh regenerates normally but is usually thickened and hyperplastic and can serve as a focus of infection.

Fin biting and pinching are regular accompaniments of keeping fish in high density. It is generally associated with territorial settlement attempts. In parrot salmon, a very distinctive pattern of dorsal fin damage, called dorsal fin rot, but actually a hyperplastic healing response, is induced as fish descend through the water column when temperatures fall in the fall. The increase in relative density in the lower levels of the retention basin, leading to the displacement of fish that may have created their own territories in the lower levels of the basin, leads to considerable antagonistic activity. It is characterized by pinching the fins, particularly the dorsal fins, of rivals. Persistent trauma leads not only to inflammatory thickening of the fin mesh, but also to hyperplasia. This creates the distinctive white tip of the dorsal fin that is so clearly seen clinically in these fish. It serves as a common route of entry for flavobacterial infection or furunculosis.

Farmed flatfish, especially halibut, are particularly prone to biting other fish's upper pectoral fin or, worse, the eye, which can be severely damaged. The condition is considered likely to occur when light reflects off the tapetum of the eye and nearby fish strike it in reflection.

8.13 Jellyfish Sting

The free-swimming marrow stages of cnidarians are responsible for severe losses of farmed marine fish in some locations and times of the year.

Blossoms of the purple-necked bird (Pelagia noctiluca) in the Atlantic and Mnemiopsis leidyi (sea nut) in the Mediterranean have caused massive losses at salmon and sea bass farms, where they have overwhelmed fish and cages with their sheer abundance. rather than any significant toxic effects. Their greater abundance and distribution is believed to be the result of overfishing from predatory species, particularly sea turtles, and possibly also rising global ocean temperatures. Other species of jellyfish are also responsible for occasional losses, including moon jelly (Aurelia aurita) and rarer species of jellyfish Siphonophores such as Solmaris corona and Apolemia uvaria.

Significant losses have been regularly associated with lion's mane jellyfish or scouter Cyanea capillata, which is capable of severe stings and can accumulate in large numbers in the coastal waters of the Atlantic and Mediterranean during certain late summer flowering years (Figure 11.13). They can be washed up against the meshes of net cages or crushed when sucked in by pumped seawater systems. Pieces of the long trailing tendrils, covered with nematocysts containing toxins, are brought into contact with the fish and pierce it to the body surface. They are also drawn into the mouth during respiration, and as the nematocysts pass the gills they discharge, causing severe edematous inflammatory lesions over large areas of the respiratory epithelium.

When fish are stung on the surface of the head or eye, their acute reaction is such that the affected animal rubs its head against the net or tank so violently that it can enucleate the orbital contents completely (Fig. 11.14). Dorsal or flank ulcerations also occur in areas where the long whiplash-like lesions associated with the release of nematocyst toxins from the tendrils appear as white lines on a darker skin color (Fig. 11.15). The losses can be very significant. In extreme cases, 200 tons of prime Atlantic salmon have been destroyed by such attacks, resulting in significant economic costs.

8.14 SUNBURN

Under natural conditions, fish avoid exposure to potentially harmful radiation from the solar spectrum by going to deeper water or the shade. However, under fish farming conditions, where stocks are kept in relatively shallow, unshaded water and at high densities, they can be exposed to significantly higher levels of solar radiation than in the wild. The condition is more severe at high altitudes and is particularly prevalent in the southern hemisphere, where depletion of the atmospheric ozone layer has meant that the sun's UV levels are much higher during the summer months than in similar regions in the north.

The proportion of the total emitted solar ultraviolet (UV = light radiation in the wavelength band 100 - 400 nm) that reaches the earth's surface is only about 3%. For convenience, the UV spectrum is usually divided into UV-A, the component that stimulates melanin pigment cells, UV-B, which triggers the sunburn response, and UV-C, the antimicrobial component.

It is a common misconception that UV-B does not penetrate more than a few millimeters into water. This is the case in water with a high particle or humus content, but can penetrate to almost 1 m in clear water, and it has been shown experimentally that reproducible sunburn can be induced in fish at depths greater than 0.5 m.

The lack of a keratinized layer in the fish skin and the presence of dividing cells in all layers makes the fish skin more easily damaged by UV-B emissions, which is made worse by the fact that the fish epidermis does not normally contain melanin-containing guard cells. Farmed fish, when exposed to UV-B in clear water, develop changes in the epidermis very similar to those observed experimentally. The lesions are usually on the most exposed areas, namely the head, dorsal fin, pectoral fins, back and tail.

8.15 LIGHTNING

Lightning strikes at fish facilities, whether direct when fish are kept in cages or in outdoor pools or ponds, or indirect when striking a building containing fish stocks, can result in significant electrical shock losses. Affected fish are usually dark in color, may all have open mouths, and usually, particularly of any size, show the characteristic vertebral fracture and associated hemorrhage in the thoracolumbar region resulting from hypercontracted bilateral myotomes. Similar lesions can be seen when large fish are electrocuted accidentally or for

slaughter. They can even be observed when exposed to excessive direct current while electrofishing.

8.16 Ulcerative skin necrosis (NDU)

The disease, now called UDN, was first observed in Atlantic salmon and sea trout in Britain between 1873 and 1911. Apparently it spread from one watershed to another as an infectious disease, and several hundred thousand fish died.

At that time the disease was not reported in any other country, but in 1964 there was a resurgence of the disease. The outbreak started in south west Ireland and spread to the rest of Ireland.

Unfortunately, diagnosing UDN is difficult and uncertain. Due to the inaccuracy of its name and the lack of an accurate diagnostic test, several other skin diseases of a variety of types, all associated with secondary infection of ulcers, have been incorrectly classified as UDN. This further confused an already complex syndrome.

8.17 ACUTE ANAPHYLAXIS

In rare cases, certain fish populations, particularly omnivores such as tilapia or carp fed a diet high in protein, may develop an acute food allergy. A few seconds after eating, much of the population goes into extreme rigidity, with all fins erect and very dark in color, and the body rigid and twitching with contractions. These fish are easy to take out of the water and their fins remain upright. If left alone, they usually recover by the next meal. The effect of this allergy strain can be experimentally prevented by premedication with chlorpromazine, but the actual trigger is unknown.

CHAPTER 9

LABORATORY METHODS

Fish are usually examined pathologically, either for diagnostic purposes when the examination is usually in some way clinically justified, or for health certification purposes. There is growing concern about the dangers of spreading potential pathogens from one area to another. Movements of live fish are considered to pose the greatest risk of such transmission, but movements of genetic material in the form of eggs or milt also pose the lowest risk.

9.1 HEALTH CERTIFICATION

Most countries with significant aquaculture industries or fisheries have strict laws regarding the import of fish and fish products. In general, for some illogical reason, these do not apply to the movement of aquarium fish, an anomaly that can be the pathway for serious disease transmission affecting a much larger area. Individual national requirements vary from country to country, but virtually all are based on the underlying principles of the Organization des epizootics in Paris, the international organization for the control of animal diseases in all animals. The rules are codified in the American and Canadian "Blue Books" which contain detailed rules for testing and certifying fish intended for import into these countries. Some aspects of these requirements are specified in the regulations of most other countries.

The number of fish to be tested for health certification purposes depends on the size of the population being studied, but since testing 100% of a population usually requires 100% culling, this option is not practical. Instead, a statistically valid sampling is carried out under the assumption of low prevalence. See des Clers (1994) for details on prevalence levels and how they are determined. Typically, the test is based on detecting a 95% probability of detecting at least one carrier fish in a population with an assumed prevalence of 2%, 5% or 10% carriers. It cannot be overemphasized that failure to isolate a specific pathogen from a population sample, regardless of sample size or assumed low prevalence, does not guarantee the absence of the pathogen.

Because of the risks associated with viral diseases such as IPN, which can be transmitted intraocularly or via milk, as well as the wide variation in detectability outside the spawning season, Roberts and Frerichs introduced the concept of 100% brood stock testing to commercial aquaculture in 1978. Originally developed for the detection of IPN in wild fish whose eggs

were exported from Scotland to Canada, it has now been adopted by many countries for import regulations for salmonid eggs.

The fish to be tested are skinned and killed, and the eggs and milk of individual pairs are fertilized. Each separately identifiable set will be kept in isolation at a spring water supply until brood stock taken during stripping for the various conditions tested is declared negative. The positives are of course destroyed.

This chapter is not intended to cover all aspects of pathology laboratory technology; there are already a number of relevant medical and veterinary textbooks for this. However, many standard techniques require modifications for use in fish pathology, and these modifications are given along with the most useful routine methods for each of the major diagnostic disciplines.

9.2 HISTOPATHOLOGY

Histology, the study of the microanatomy of specific tissues, has been studied since the first cellular studies in the mid-19th century. Since then, considerable developments have taken place in all aspects of cell biology, with the result that today many new and sophisticated histological techniques developed recently for the mammal -histologists are now available to the fish histopathologist.

Before satisfactory histological sections can be obtained from biological material, careful attention must be paid to its preparation. The very rapid rate of autolysis of fish tissue compared to that of homeotherms means that they must be handled quickly to avoid degenerative changes in the sample that make definitive diagnosis unreliable or impossible.

9.3 SAMPLING PROCEDURES

Only freshly killed or dying fish should be considered for satisfactory histological preparations.

External lesions

Careful post-capture management is essential for most external lesions, as the epidermis is easily eroded by bony fish.

For optimal preparation, fish should be taken out of the water with hooks or fine-mesh nets and quickly transferred to a suitable general anesthetic container or alternatively beheaded. The

fish should then be handled with tweezers or, if too large, by the tail or fins if these areas are not being examined. Lesions should be excised or, if the entire specimen is retained, multiple deep parallel incisions made along the length of the body wall, from nose to tail, to allow immediate access to the fixator. A little care at this stage will be rewarded by the quality of the information ultimately obtained.

internal lesions

If the lesions are internal and the whole fish is to be preserved, it is important to open the full length of the body cavity, usually by incising along the ventral midline. The intestines and swim bladder should be gently manipulated and each organ incised at least once to allow maximum penetration of the fixative. Ideally, however, the organ or lesion to be examined should be carefully excised from the body, cut into blocks < 1.0 cm 3 and placed in a volume of fixative at least 20 times the volume of the tissue.

9.4 MOUNTING: IDEAL MOUNTING

The fish researcher is presented with a bewildering array of tissue fixatives, each with its own advantages and disadvantages. Good fixation is fundamental to a satisfactory histological preparation and its importance cannot be overstated. If the fixation is unsatisfactory, the final product will reflect this directly. The main goal of fixation is to preserve the morphology of the tissues in a state as close as possible to that during life. This involves inhibition of both autolysis - the "self-destruction" of tissues by intracellular enzymes released from their normal membrane - a site bound after death - and putrefaction - the effects of bacterial tissue breakdown. In tropical conditions, it's important to keep the hairspray cool.

9.5 FIXative commonly used in fish pathology

Probably the most widely used fixative in any histology laboratory is formaldehyde (H.CHO) (Steedman 1976), a water-soluble gas supplied in a concentrated form at 40% by weight. In concentrated solution, formaldehyde often becomes cloudy during storage due to the formation of paraformaldehyde. Warming the solution or adding a small amount of NaOH will aid in the depolymerization of paraformaldehyde. Alternatively, it can be removed by filtration. Formaldehyde is not suitable for fixation in its concentrated form, but is an integral part of various compound fixatives, usually in combination with diluents such as tap water/distilled water, buffered saline, or physiological saline. The main disadvantage of formaldehyde as a

fixative is not its effect on tissues, but its unpleasant vapor, which can cause extreme irritation of the respiratory tract and eyes. In addition, it can sensitize the skin and cause "formalin dermatitis", so proper precautions should be taken to protect hands and face when using this substance.

All formaldehyde, regardless of its purity, is acidic when purchased, typically in the pH range 3 to 5. Care should be taken to check the final pH of formalin-based fixatives.

Fixative with formaldehyde phosphate buffered formalin

40% formaldehyde	100 ml
Tap/distilled water	900 ml
$NaH_2PO_4H_2O$	4 g
Na_2HPO_4	6 g

Probably the most satisfactory compound fixative available to the fish pathologist. It preserves textural detail with minimal distortion. Fixing time is not critical. Although most tissues are properly fixed in 8 to 24 hours for routine diagnostic purposes, they require significantly longer fixation time if optimal preparation is to be achieved.

'Susa' by Heidenhain

Mercuric chloride	45 g
Sodium chloride	5 g
Trichloracetic acid	20 g
Acetic acid	40 ml
Formalin	200 ml
Distilled water	800 ml

Although this fixative is generally less suitable than phosphate-buffered formalin for routine use, it is probably the best available for rapid fixation of small pieces of tissue. It contains mercuric chloride, a protein precipitating agent that penetrates and hardens quickly. The penetration speed decreases after 2 to 3 minutes, so the tissue blocks must not exceed 5 mm 3, otherwise there is a risk of overfixation at the periphery and insufficient fixation towards the center.

Tissues with a thickness of 2-3 mm are sufficiently fixed in 3-5 hours. This setting time should not be exceeded as this will result in excessive hardening and fading of the block. As the mercuric chloride component is a highly toxic and corrosive chemical, caution should be

exercised in its use. Fixatives containing mercuric chloride should not be stored in containers with metal lids. The sample should be placed in 95% alcohol (methylated spirits) for processing as immersion in an aqueous solution will cause excessive swelling of the connective tissue present. As with any mercuric chloride fixer, the fixed sections in "Susa" must be treated to remove any black precipitate of mercury, which can easily be confused with teleost melanin. There are two methods of doing this: pretreating the block (1) by incorporating iodine into the alcohol baths during the dehydration process, or (2) treating individual sections prior to staining.

Cytoplasmic and nuclear staining tends to be enhanced after ingestion of mercuric chloride, particularly in fish, in parenchymal organs such as the liver or kidney. It is often found that sections initially fixed in formalin-based fixatives benefit from a pretreatment with a saturated aqueous solution of mercuric chloride, which improves the intensity and contrast of the final stained specimen. However, 'Susa' has two major disadvantages. The high cost of the mercuric chloride component makes it an extremely expensive solution for regular use, and mercury solutions are usually very difficult to dispose of due to sewerage regulations.

Bouin's liquid

Saturated aqueous picric acid	75 ml
Formalin	25 ml
Acetic acid	5 ml

Although Bouin's liquid is widely used in fish pathology due to its rapid penetration with little shrinkage or distortion, it causes partial destruction of red blood cells and has a swelling effect on collagen fibers. However, these disadvantages are somewhat compensated for by the later observed light coloration, especially with trichrome methods. Glycogen is brought out fairly well, although replacing the aqueous component with 95% alcohol (gender fluid) gives better conservation of glycogen. Small pieces of fabric 2-3 mm thick are fixed in 2-3 hours; larger blocks require up to 24 hours.

Picric acid precipitates proteins and combines with them to form picrates, some of which are water soluble, so the bound material must be converted directly to the alcohol to prevent these soluble picrates from dissolving in water. Yellowing of tissues by picric acid may be of benefit in small specimens, but should be removed from sections with 2.5% sodium thiosulfate before using aniline stains, otherwise a precipitate will form. Bouin's is not recommended for fish skin

preparations as it makes scales even more difficult to cut and the differential swelling is extreme.

Carnoy's liquid

Absolute alcohol	60 ml
Chloroform	30 ml
Acetic acid	10 ml

Although rarely used in fish pathology, Carnoy is probably the most rapidly penetrating tissue fixative. This rapid penetration is not without drawbacks. Carnoy fixed tissues show considerable shrinkage and many cytoplasmic elements are destroyed. Shrinkage can be reduced somewhat by fixing at 0°C. Fixation of a 5mm thick block is typically completed in 1-2 hours, small biopsy specimens in 15 minutes. The fixatives listed above are the most commonly used in histology laboratories; However, Zenker and Helly fluids work well when applied to tissues such as the brain, pituitary and pancreas. However, on fish skin they cause significant swelling of the dermis and considerable deformation of the scales with subsequent destruction of the various layers of skin, making them unreliable for use on external tissues. Although the primary goal of any fixative is to preserve tissues in as realistic a form as possible, when choosing a fixative it should be borne in mind that their mode of action can differ significantly; for example, mercuric chloride and picric acid-based fixatives generally impart a lighter color to the final product, but fixation times are quite critical, with over-fixation rendering the preparation unsuitable for diagnostic purposes. Formalin-based fixatives, on the other hand, do not normally pose such a problem, with satisfactory preparations often being obtained several years after initial fixation.

9.6 DESCALING

Although widely used in mammalian histology (Culling 1963), decalcification techniques are less commonly used in fish laboratories. The main goal of decalcification is to remove calcium ions from the bone components of the specimen without damaging the other components to facilitate sectioning. Decalcification methods inevitably cause some degree of cell damage or have an adverse effect on subsequent staining. This is particularly the case with fish tissue, since the compact, medullary nature of fish bones necessarily means that exposure to the decalcifying agent is often much higher than the surrounding tissue can tolerate. The most important requirement for satisfactory decalcification is proper fixation of the tissue before

exposure to the decalcification fluid. Cook and Cohen (1962) showed that tissue damage during acid decalcification is about four times greater when fresh tissue is used.

Scaling methods vary widely, and as with pinning, no single solution is ideal in all respects. Basically, the processes used can be divided into two groups: (1) acidic decalcifying agents and (2) chelating agents.

Acid decalcification

There are many methods for removing calcium salts by acid solutions (Wahtola & Owen 1970). Most are fairly unsuitable for use on fish due to their pungent action, but where an alternative treatment is not possible, the use of 8% formic acid in distilled water may be satisfactory. The solution must be renewed frequently (every 8 to 12 hours) and when decalcification is complete, the tissue must be transferred to 70% alcohol. Another acidic method involves the use of trichloroacetic acid as a freshly prepared 5% aqueous solution. This decalcifies fairly quickly with reasonably good subsequent staining and, as with formic acid decalcified tissue, the material can be transferred directly to 70% alcohol prior to dehydration. vs

healing method

The most satisfactory method for decalcifying fish tissue is to use ethylenediaminetetraacetic acid (EDTA), a chelating agent. Since no gas bubbles are created with this technique, tissue tearing is less likely. The solution is active at neutral pH, so subsequent staining is not affected.

EDTA descaling solution

EDTA	250 g
Distilled water	1750 ml

The solution is adjusted to neutral pH by adding NaOH. Although it gives excellent results when used successfully, this method has two disadvantages: (1) it is relatively slow compared to acidic decalcifiers (samples can take several days to decalcify) and (2) the chemical check at the end - the scaling point is particularly unreliable for this method.

end point of decalcification

Determination of the endpoint of decalcification is best done by X-ray examination. However, many diagnostic laboratories do not have access to such a facility and must therefore rely on chemical tests that determine the presence or absence of calcium salts using the following method. Strong ammonia is added to 5 ml of the descaling liquid to be tested until the sample is almost neutral. 1 ml of 5% sodium or ammonium oxalate is added. The turbidity of the solution indicates the presence of calcium. The absence of turbidity after a period of 5 minutes indicates that the liquid is calcium free. This indicates that the calcium ions have been removed from the tissue and further processing can be carried out. In general, therefore, decalcifying agents are useful in fish histology. However, there are strict restrictions on their use in the seed coat. It is often believed that decalcification of fish scales results in better skin preparation; this is rarely the case, although the scales contain calcium salts. The resulting swelling and rupture of the tartaric protein after treatment with decalcifying agents often renders the resulting preparation quite unsuitable for further study.

Processing

Before the treatment, it is important to ensure that no special post-fixation is required, such as direct transfer to an alcoholic solution as with Bouin's liquid or prolonged washout times as with Zenker's Bouin's liquid. Before a fixed material can be used for microscopic examination, it must be impregnated with a medium that provides sufficient support to allow thin sections (5–7 μm) to be cut. The substance of choice is usually paraffin wax, although other media such as celloidin or gelatin can be used. Treatment of the fixed tissue involves dehydration by increasing concentrations of alcohol, "clarification" in a wax - a miscible agent such as xylene or chloroform - and finally impregnation with wax.

dehydration

In order to infiltrate with wax, all water must first be removed from the fixed tissue (see Table 12.1). It is common to start the dehydration with a 50-70% dilution of alcohol in water to avoid the distortion that would occur if a direct transfer to absolute alcohol were made. Especially with hard tissues such as skin, it is beneficial to include multiple 8% Methyl Alcohol-Phenol (66OP) baths in the treatment regimen, as phenol has a softening effect on tissues. The tissues are finally transferred to absolute alcohol baths (74 OP) for complete dehydration.

Table 12.1 Schedules for processing fish tissue for histopathology.

Schedule A (manual processing)
1. Fix tissue. 2. Wash in H_2O desirable. 3. 70% alcohol, 4–8 hours. 4. 90% alcohol, 4 hours or overnight. 5. Absolute alcohol (74 OP) I, 2 hours. 6. Absolute alcohol (74 OP) II, 3 hours. 7. Absolute alcohol (74 OP) III, 3 hours. 8. Chloroform, overnight. 9. Wax I, 2 hours. 10. Wax II, 2 hours. 11. Wax III, 2 hours.

Schedule B (automatic tissue processor)
1. 50% alcohol, 1 hour. 2. 80% alcohol, 2 hours. 3. 8% phenol meths I, 2 hours. 4. 8% phenol meths II, 2 hours. 5. 8% phenol meths III, 2 hours. 6. Absolute alcohol (74 OP) I, 2 hours. 7. Absolute alcohol (74 OP) II, 2 hours. 8. Chloroform I, 1 hour. 9. Chloroform II, 2 hours. 10. Wax I, 2 hours. 11. Wax II, 3 hours. 12. Wax III, 3 hours. This schedule has been found to be particularly useful for difficult tissues such as fish integument.

Schedule C (rapid manual process)
1. Fix in Carnoy's fluid, 30–60 minutes. 2. Absolute alcohol (74 OP.) I, 30 minutes. 3. Absolute alcohol (74 O.P.) II, 30 minutes. 4. Absolute alcohol (74 O.P.) III, 30 minutes. 5. Xylene until tissue appears transparent. 6. Wax I, 30 minutes. 7. Wax II, 30 minutes. 8. Wax III, 30 minutes.

Note: These times are guidelines only and may need to be changed to accommodate specific fabrics.

clearing

Since alcohol is immiscible with paraffin wax, the fabric must first be treated with an agent that is miscible with both substances. There are several such reagents in common use, of which xylene, chloroform and toluene are most preferred. Xylene is probably the most commonly

used and has the added benefit of making the block transparent after cleaning is complete. Its effects are rapid but tend to weaken tissues with prolonged exposure. On the other hand, chloroform, although the most expensive of the clarifying agents, does not seem to have the same hardening effect as xylene and is found to be more satisfactory with fish tissue. However, it should be noted that chloroform has a very low boiling point (60°C). If blocks are transferred directly to wax at a temperature in excess of 59°C, tissue rupture can occur with catastrophic results. Almost all cleaning agents are volatile and in many cases toxic and flammable. They must therefore be handled with care and stored in sealed containers. More recently, citrus extracts have found customers as clarifying agents in many histological laboratories. They have the distinct advantage of being non-toxic by inhalation or dermal absorption and are also biodegradable.

Impregnation and wax coating

The function of wax impregnation is to provide a firmly supported block for cutting. Ideally, the wax should have the same hardness as the tissue to be examined; However, this is rarely achieved due to the wide variety of consistencies found in fish tissue. The hardness of wax is given by its melting point (MP); The harder the wax, the higher the melting point. The most commonly used waxes are in the 54-58°C range. There are many choices available to the histologist, and the final choice depends largely on the tissue being examined.

9.7 BLOCKING PROCEDURE FOR WAX IMPREGNATED FABRICS

1. Glass and metal molds should be coated with glycerin to prevent wax from sticking to the surface.

2. The melted wax is then poured into the mold; Within seconds, the wax solidifies at the bottom of the mold.

3. The tissue is transferred with heated tweezers and aligned in such a way that the side to be cut is firmly anchored in the hardening layer.

4. The shape is identified by labeling it with the block number.

5. When a thin layer is hardened on the outer surface, the mold is quickly immersed in cold water, which will speed up the hardening of the entire block.

6. After complete solidification, the block is removed from the mold and mounted on wooden blocks for microtomy.

9.8 BLOCK COOLING

When cutting blocks, there is a distinct advantage in keeping the blocks cold, possibly by placing them on ice for several hours. This method results in a firmer block and is particularly useful for difficult objects like fish skin. CRYOSTATIC SECTIONING The optimum temperature most suitable for making cryostatic sections depends on the type of tissue to be sectioned, fixed or unfixed, and the subsequent thickness required. A cabinet temperature of -15°C to -20°C has generally been found satisfactory for most tissues studied.

1. Secure the tissue on the slide by pipetting water onto the surface of the slide and quickly placing the sample in situ before freezing. To create a firm cutting base, it is beneficial to pipette water into the sides of the block to build up a firm matrix. This also prevents the block from detaching from the specimen holder during sectioning.

2. The area of the block is cut in the same way as for paraffin sections.

3. Once the cryostat guide plate is in place, sections are cut to the required thickness (typically 15–20 μm).

4. The cut section should lie flat on the knife blade with the baffle in the down position. A clean, room temperature slide is placed flat on the section, which is then transferred to the slide. Section adhesives are generally not required for cryostat sections.

5. Sections can be processed unfixed or, if necessary, soaked in 5% acetic acid in absolute alcohol (74 OP) for 1-2 minutes before proceeding with the staining technique.

9.9 IMMUNOFLUORESCENCE

This technique has particular advantages for the experienced user in the rapid diagnosis of bacterial and viral infections and the localization of viral or bacterial antigens in tissues. Bullock and Stuckey (1975a,b) developed an immunofluorescence test for the rapid specific diagnosis of corynebacterial nephropathy, a condition that can present significant diagnostic difficulties.

1. Swabs or cryostat sections from suspect tissue are fixed in acetone for 5 minutes, then washed in phosphate buffered saline (PBS) (0.138% NaH 2 PO 4 H 2 O in saturated saline, pH 7.2).

2. One drop of commercially prepared rabbit anti-KD bacterial antiserum is placed on the tissue and incubated at 37°C for 30 minutes.

3. The antiserum is washed thoroughly with PBS and the slide washed three times in PBS.

4. After air drying, one drop of fluorescein isothiocyanate labeled anti-rabbit globulin (goat anti-rabbit) antiserum is placed on the tissue, incubated for 30 minutes and then washed with PBS.

5. After several additional washes, the sample is ready for analysis. It is important to use known negative controls for this technique to avoid false positive results.

Samples should be examined under ultraviolet light, preferably by Ploem's method. The light source is usually a mercury vapor lamp with blocking, excitation and blue interference filters. Further details should be consulted from Nairn (1977) before engaging in this versatile and highly specific technique which can be developed for many other purposes in fish pathology, but the interpretation of which is somewhat subjective in all but the most experienced hands.

9.10 COMPLETE FIXATION TEST

This test is based on the consumption (fixation) of complement (a complex of non-antibody serum proteins) when an antigen-antibody reaction occurs. The test determines the presence and amount of viral antigen. The test method measures whether complement has been used in the antigen-antibody reaction by adding an indicator system. Sheep erythrocytes sensitized by mixing with haemolysin (antibody against sheep erythrocytes) are used as the indicator system. In the presence of complement, hemolysin will lyse erythrocytes, but cannot if complement has been fixed in the first antigen-antibody reaction.

CHAPTER 10

CONCLUSION

Overall, we can consider proteomics as a very promising tool for the study and diagnosis of fish pathology, allowing a more holistic approach to the processes of pathogenesis and providing important insights into pathogen identification and characterization of virulence mechanisms and host-pathogen interactions, that shed light on new stress response pathways. and previously unknown host physiological responses.

However, the application of proteomics in fish farming is still in its infancy and limited to certain sequenced organisms. Recent advances in the definition of aquaculture proteomes and large datasets of diseased fish and fish pathogens will stimulate the use of proteomics techniques in aquaculture and lead to exciting new discoveries in the field.

But one of the most promising and interesting areas, which we believe will be the future trend to better understand the response of fish to pathogens, is the study of the interaction between the holobiome, host and pathogen, with strong potential for more detailed and integrated ones new discoveries. Knowledge of the pathogenesis of fish.

Author Contributions:

MM: Conceptualization, Methodology, Formal Analysis, Research, Writing - Original Draft, Writing - Revision and Editing Visualization. DS: Writing - Original Draft, Writing - Revision and Editing. APF and MC: Writing - Original Draft. CRdM: Writing - Original Draft, Visualization. RC: Writing - Original Draft, Writing - Revision and Editing. PR: project management, financing acquisitions, conception, writing – editing and editing, supervision. All authors have read and accepted the published version of the manuscript.

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