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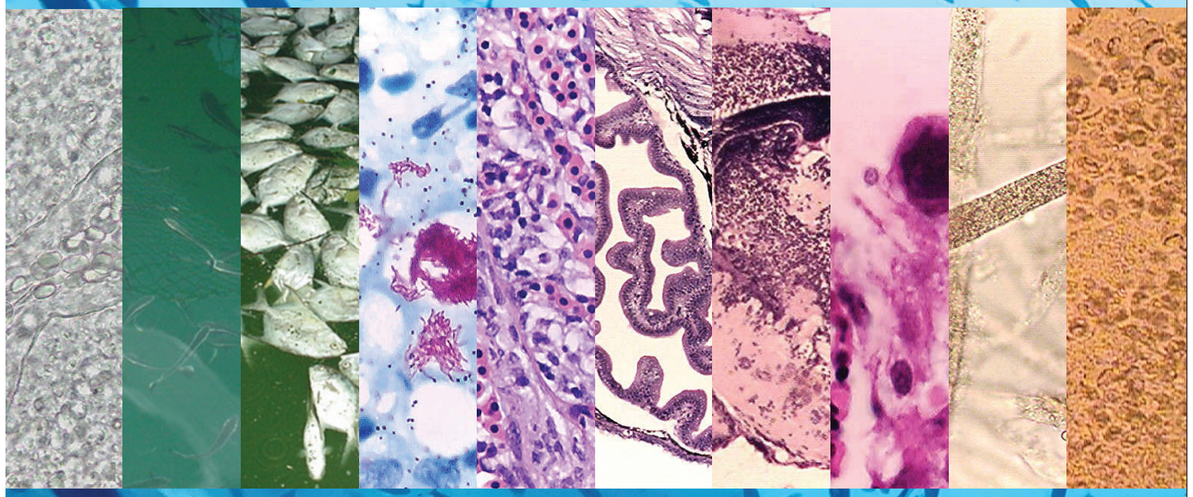
Pathology of Cultured Fish in Hong Kong

Front Cover



Pathology of Cultured Fish in Hong Kong

"Give a man a fish and you feed him for one day. But teach him to fish and you feed him for life."
Chinese Proverb

For my family,

Rosalind
Samantha and Wesley

Your love and understanding makes the cup of life full with God's grace.

FOREWORD

This text comes out of initial work done by the Fish Diseases Section, Veterinary Laboratory Division of the Department of Agriculture, Fisheries and Conservation (AFCD), Hong Kong Special Administrative Region (HKSAR) Government from late 1999 to 2003. Its purpose is to provide a basis for the ongoing service provided to the Hong Kong aquaculture sector in the management of fish diseases. Aquaculture is an expanding primary production globally and is posed to fill the supply of fish and shellfish food requirements as fisheries harvest has plateaued. However, diseases continue to be a key threat to sustainable production. The availability of fish health services is vital for managing such existing and emerging diseases. This book describes the common fish diseases in Hong Kong from the perspective of a veterinary fish pathologist. It is intended to be a basic guide for the laboratory pathologist, the field extension veterinarian or fisheries biologist and extension of the investigation of common fish diseases endemic to Hong Kong.

The efforts of many colleagues are involved in this project beginning with laboratory staff members at the Tai Lung Veterinary Laboratory who have produced the material for this book. My heartfelt thanks go to the staff of the Fish Disease Section: Walter Wong, Charles Chan, Leona Lam, Thomas Leung, Cynthia Liu, Laura Yuen, Jeannie Ng, Carrie Chan, John Lau and Mok Kam Chiu. You have worked exceptionally well as a team with a deep commitment to the task of helping farmers with their fish health challenges. Leona Lam, who contributed much to the bacteriology of the fish cases, deserves a special mention in memory of her unexpected passing following a period of illness. Much appreciation is due to Drs. Trevor Ellis, Kitman Dyrting, Geraldine Luk and William Wong for their support to the Fish Diseases Section. I also acknowledge the encouragement of Pamela Li and Bernard Chan through their extensive laboratory technical experience.

The support of colleagues of the Fisheries Branch and their generosity in sharing their considerable aquaculture industry and fisheries knowledge are much appreciated. In particular I thank Aaron Leung Chi Fai, Dr. Patsy Wong, Dr. Jim Chu, Minna Wong, Louise Li, Vivian Au, Anna Situ and Dr. Joanne Lee. Editorial assistance was generously provided by Jim, Anna, Joanne, Yvonne Cheung and Dr. Ling Leung in the refinement of the manuscript.

With the encouragement of Dr. Leslie Sims and Dr. Kwei Kin Liu, this work was made possible and my thanks is their due.

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A special thanks goes to Dr. Barry Munday (University of Tasmania) who provided great help in my learning of fish diseases, pathology and histopathology even during his period of illness just before his passing. Much debt is owed to Dr. Judith Handlinger (Tasmania) and Dr. Brian Jones (Department of Fisheries, Western Australia) for valuable expert technical and editorial input into the manuscript, together with generous and patient guidance.

Of course, fundamental to the completion of this work is my family - Rosalind, Samantha and Wesley through the months of absenteeism when I would have given up but for their love, belief and prayers.

This work is dedicated with thanks to my Lord Jesus Christ, who gave me strength, direction and wisdom for each facet of the work. “Give thanks to the LORD, for he is good; his love endures forever. Whoever is wise, let him heed these things and consider the great love of the LORD.” Psalm 107: 1,43.

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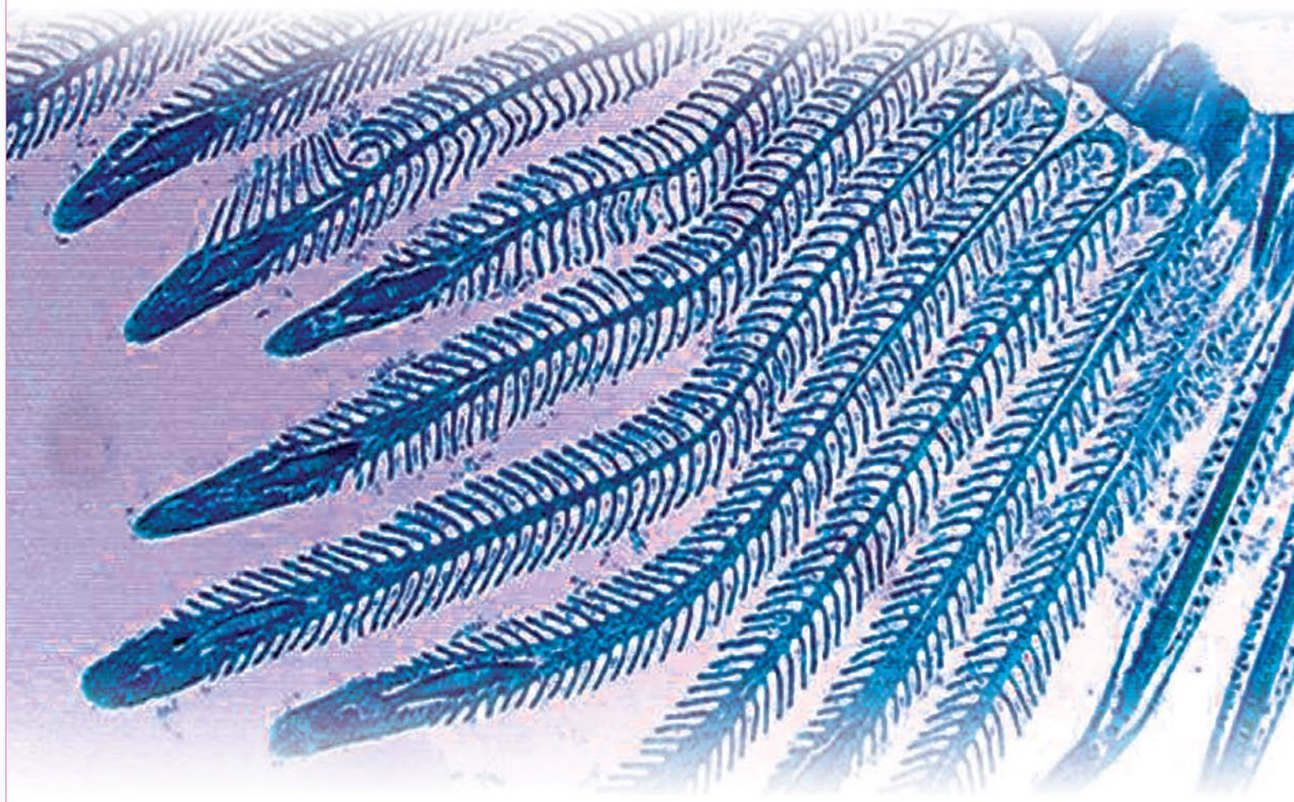
Certified Fish Pathologist (American Fisheries Society – Fish Health Section)

17 September 2013

CONTENTS

PART 1 – NORMAL FISH BIOLOGY



PART 1 – NORMAL FISH BIOLOGY

Fish Anatomy

Fish are created to function in an aquatic environment. Their anatomy reflects the ability to cope with the physiological demands of a wide variety of aquatic environments. Fig. 1 shows the external anatomy, while Figs. 2-4 show the internal anatomy of teleost fish.

Integument

The skin of fish (Fig. 5) is a complex organ consisting of an epidermis with mucous cells, dermis, scales and secreted mucus together with a wide variety of pigmentation. As scales are embedded in the dermis, loss of scales represents damage to the epidermis and provides egress for pathogens. Mucus is also of interest, it is not only used to facilitate water flow by minimising friction, but it is immunologically active, has an osmoregulatory role and probably other functions as well. Skin types vary with the species of fish in terms of size and shape of scales, thickness of the skin and degree of slipperiness. In marine fish like grouper and pompano, the scales are harder than in freshwater fish. This is probably due to the amount of calcium in the scales and it reflects the nature of the habitat. The mucus also varies in amount and viscosity between species. Goldfish produce fairly fluid mucus whereas marine amberjack can have very sticky mucus. The salinity in which fish live can affect the activity of mucus or goblet cells, being mediated by prolactin, cortisol and growth hormone (Marshall, 1979). The biochemical properties of mucus also vary with salinity changes and the presence of ectoparasites e.g., amoebae (Roberts and Powell, 2005). This may influence the level of protection the mucus affords to the fish against osmotic gradients and pathogens. Parasites are often found in the mucus of skin scraping and the shedding of excess mucus is an effective defence against the establishment of low numbers of skin parasites. In some species like the Discus, specialised mucus of the parent fish is a source of food for Discus fry. The Discus mucus contains a higher protein content and essential amino acids lysine and phenylalanine, which provide necessary nutrition to the larval fish (Chong *et al.*, 2005). Normal skin should have a glistening appearance and the amount of mucus should be evenly spread over the body of the fish. The scales should be intact and the colour should be of an even hue with no areas of unnatural darkening. Black fish or mottled fish may be normally dark; hence species differences should be appreciated. Note that a euthanased fish will often be darker as well as sick or blind fish because skin colour is under visual and neural control.

Muscle

Fish muscle (Fig. 5) is found on either side of the flank lying directly under the skin and contains little if any fat in both marine (grouper) and freshwater (tropical) fish. However, some fat may be present under the skin and within the muscle of some fish species and particularly it is mostly white muscle but also contains a band of red muscle, which normally runs along the lateral line region. This can be prominent in mackerel (*Trachuridae* and *Scombridae*). Fish muscle occurs in blocks with a "V" shape, allowing for sinusoidal lateral flexure. The texture of the muscle should be firm and not watery. Watery muscle is encountered if the skin barrier has been breached and oedema or infection has set in. The white muscle should have a slight translucent appearance to it and not be an opaque white. The degree of muscling gives an indication of the body condition of the fish. Catabolic, diseased fish may have a sunken-in appearance of the muscle mass around the head, leading to a pinhead shape to the fish where the body muscle mass of the fish is reduced compared to the head. White muscle has fewer blood vessels than red muscle; therefore any area of increased vascular supply should be investigated.

Gills

Fish gills (Fig. 6) are a very important interface between the internal and external environments of the fish. Normal gills will have a clean, red colour with a glistening appearance, which is an indication of normal blood circulation, mucus function and absence of particulate debris. Gills that are dirty looking, pale or with excessive mucus are diseased. There are two pairs of gill sets, one either side of the opercular cavity of fish. Each set has four gill arches. The gill filaments sit on a bony/cartilaginous gill arch, which at the anterior curvature carries the tooth-like gill rakers. The gills are made up of larger primary filaments (synonym = filament) and smaller secondary filaments (synonym = lamellae). This design creates a very large surface area for effective respiration, osmoregulation and excretion. In polluted aquatic environments, the gill is usually one of the first organs to be damaged due to the delicate filaments being in direct exposure to water-borne contaminants.

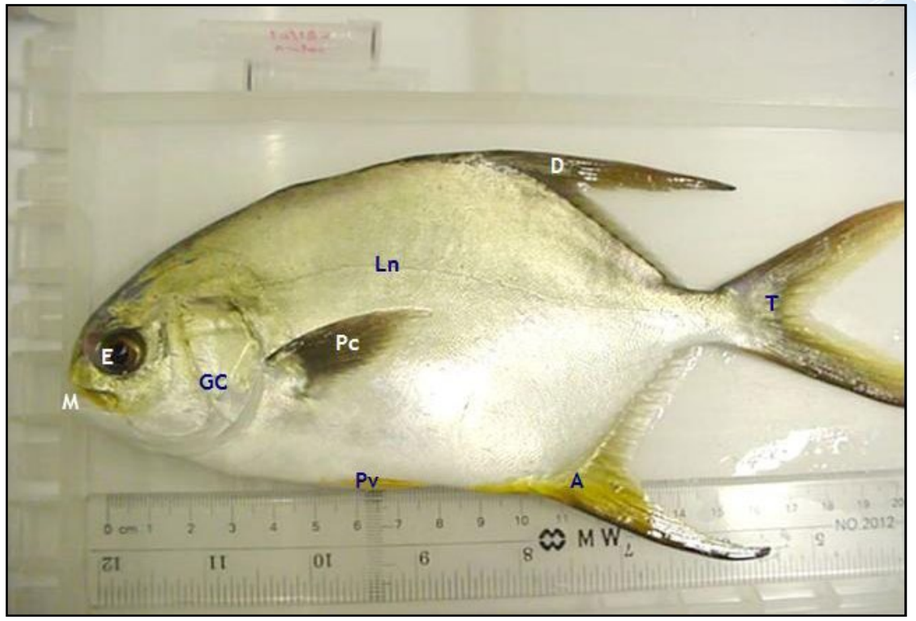


Figure 1. External anatomy of pompano (*Trachinotus blochii*), a marine fish species: lateral line (Ln), pectoral fin (Pc), dorsal fin (D), pelvic fin (Pv), anal fin (A), tail fin (T), gill cover (operculum) (GC), eye (E) and mouth (M).

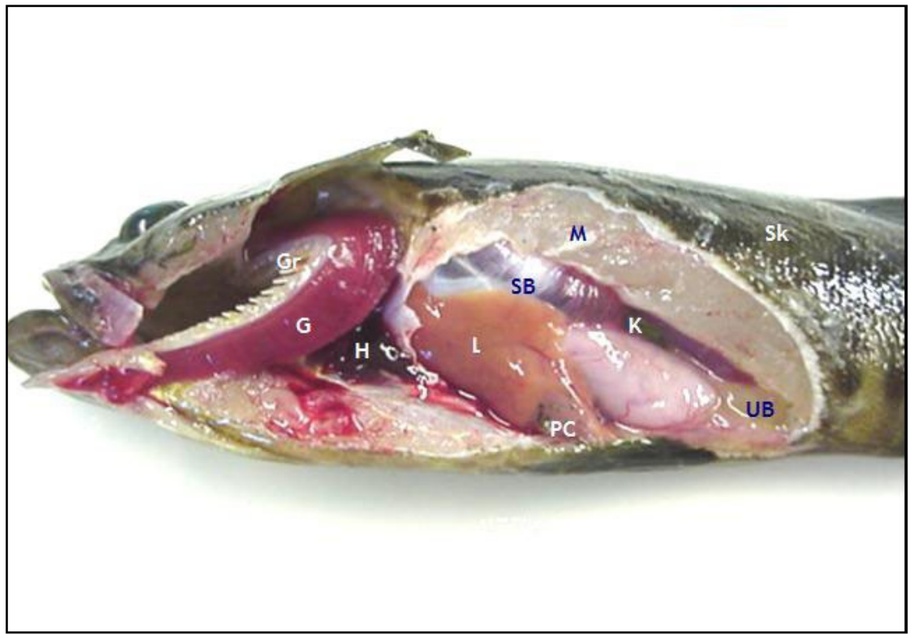


Figure 2. Anatomy of green grouper (*Epinephelus coioides*): gill filaments (G), gill rakers (Gr), heart (H), liver (L), swim bladder (SB), urinary bladder (UB), pyloric caeca (PC), kidney (K), muscle (M), and skin and scales (Sk).

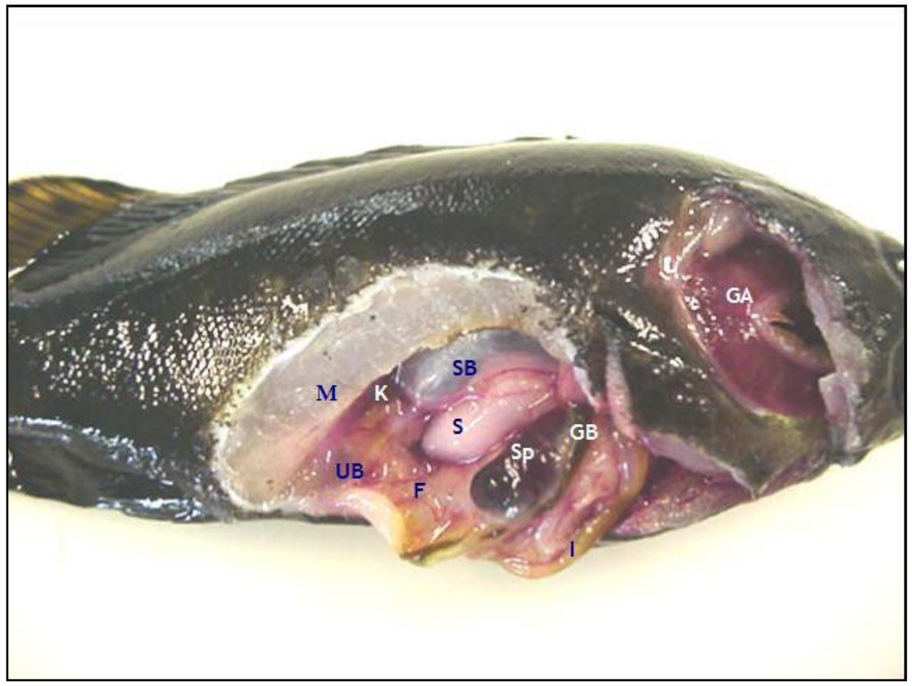


Figure 3. Anatomy of giant grouper (*Epinephelus lanceolatus*): spleen (Sp), stomach (S), gall bladder (GB), intestine (I), peritoneal fat (F), gill arch (GA), swim bladder (SB), urinary bladder (UB), kidney (K) and muscle (M).

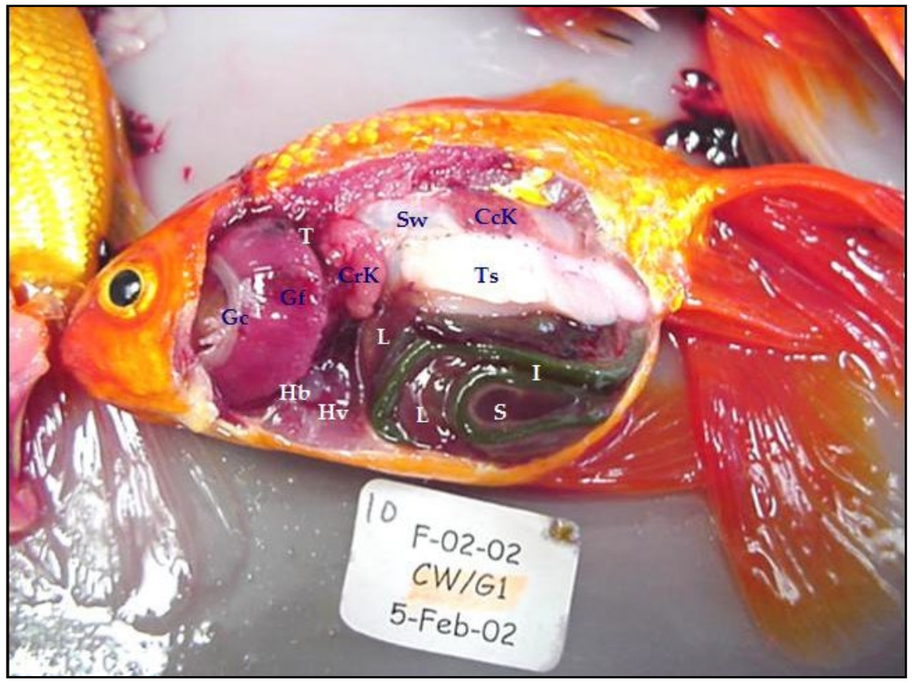


Figure 4. Internal anatomy of goldfish (*Carassius auratus)*: cranial kidney (CrK), caudal kidney (CcK), thymus (T), gill filaments (Gf), gill arch cartilage (Gc), heart: bulbus arteriosus (Hb), heart ventricle (Hv), swim bladder (Sw), intestine (I), spleen (S), liver (L) and testes (Ts).

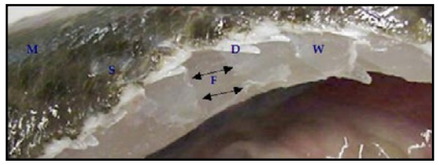


Figure 5. White muscle of the flank in green grouper (*Epinephelus coioides*): white muscle (W), scales (S), dermis (D), fascial planes (F) and mucus (glistening) (M).

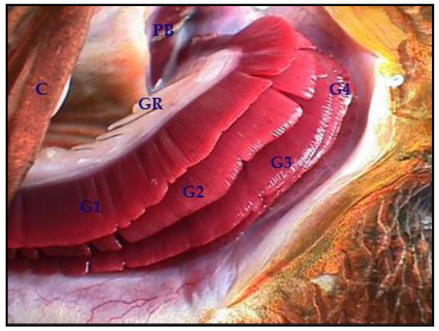


Figure 6. Normal well vascularised gills, mangrove snapper (*Lutjanus argentimaculatus*): gill arches 1 to 4 (G1-4), gill rakers (GR), pseudobranch (PB) and gill cover (operculum) (C).

Pseudobranch

This organ is located in the dorsal gill cavity. It is well vascularised and thus red in colour. The pseudobranch (Fig. 7) is of two types: those covered by a mucous membrane such as in grouper and goldfish, and those with a free pseudobranch that has direct water contact. Several functions have been proposed for the pseudobranch. These include monitoring of blood parameters such as oxygenation, osmolality, blood pressure and carbon dioxide levels. In some fish species, the pseudobranch supplies blood to the choroid of the eye and the rete mirabile of the swim bladder. The pseudobranch has no respiratory function in species where it is covered and receives oxygenated blood from the gills. Damage to this organ is thus expected to produce impaired homeostatic regulation.

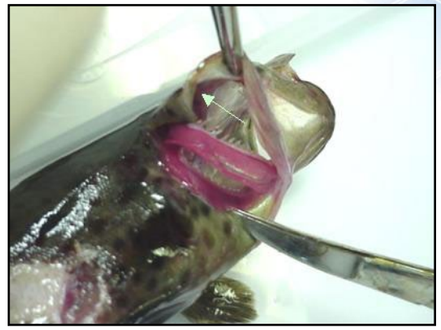


Figure 7. Pseudobranch (arrow) in the dorsal gill cavity, green grouper (*Epinephelus coioides*).

Heart

This is located in a pericardial sac just behind the gills and has a ventricle, atrium, bulbus arteriosus and sinus venosus. The heart (Fig. 8) receives venous blood from the organs via the sinus venosus through the atrium and is pumped by the ventricle through the bulbus arteriosus into the gills. The ventricle has a compact muscular outer layer and a trabecular interior. Valves exist in the sino-atrial, atrio-ventricular and bulbus-ventricular junctions to prevent backflow of blood. The bulbus arteriosus is normally white and consists of elastic collagen to dampen the blood pressure pulse from the heart while maintaining steady blood flow. The pericardium and pericardial sac are normally very thin having a small amount of serous fluid in the pericardial cavity, which separates the two membranes; however excessive fluid or haemorrhaging into this space can cause cardiac tamponade. In healthy fish, the colour of the ventricle is a deep red and the blood that flows from the heart to the gills via the ventral aorta is un-oxygenated (dark red) blood. The ventricle in some fish is supplied with oxygenated blood via coronary vessels that course over the outside of the ventricle supplying the compact muscle while the spongy myocardium is supplied from blood in the cardiac lumen.

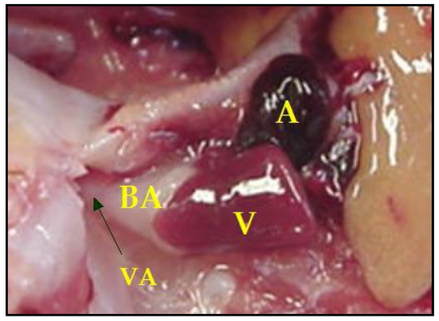


Figure 8. Russell's snapper (*Lutjanus russelli*) heart: ventricle (V), bulbus arteriosus (BA), atrium (A) and ventral aorta (VA).

Digestive Tract

The digestive tract (Figs. 9-10) begins at the mouth, continues into the pharynx, oesophagus, stomach and intestine, and ends at the anal opening. The digestive tract of fish is created to accommodate the type of diet suitable for the species. Groupers being carnivorous have strong teeth, a muscular, acid producing stomach and a simple, short intestine. The intestine has extensions called the pyloric caeca. In contrast, herbivorous fish like the goldfish have pharyngeal 'soft' teeth, and do not have an acid stomach but instead are equipped with a long and thus coiled intestine to be able to digest plant nutrients. One of the signs of disease is an empty digestive tract due to inappetence. However fish kills have also occurred due to a full stomach because fish require increased oxygen to digest and assimilate food, and hypoxia occurs often after feeding when the dissolved oxygen is inadequate in the culture environment. Fish do not have discrete salivary glands, and the function of saliva is replaced by that of mucus secreted by pharyngeal mucus glands. In healthy fish the digestive tract should contain some ingesta and digesta. There should be some fluid associated with the digesta which in marine fish (groupers) is normal as they drink in seawater in order to osmoregulate.


Figure 9. Mud carp *(Cirrhinus molitorella)* – a herbivore with a very long and coiled intestinal tract.

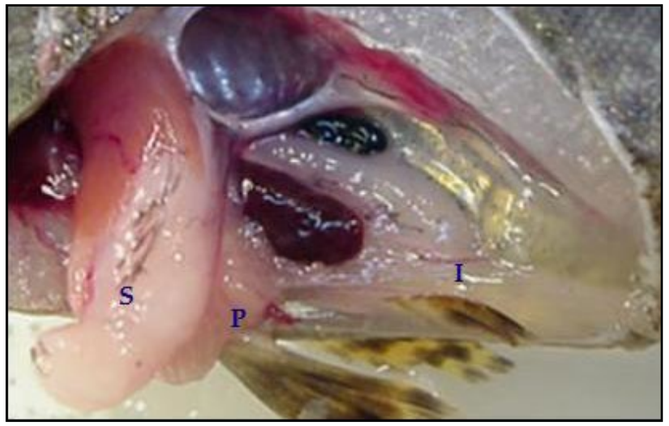


Figure 10. Tiger grouper (*Epinephelus fuscoguttatus*) – a carnivore: stomach (S), pyloric caeca (P) and intestine (I).

Liver, Gall Bladder and Pancreas

The liver is quite obvious given its size and normal location in the cranial abdomen; however in goldfish, it can be quite dispersed into smaller lobules amongst the intestinal coil (Fig. 11a). Its colour is very variable depending on the diet (Fig. 11b) but also reflects the health of the fish. A fish fed on a high-fat diet will have a yellow to almost pale creamy colour – quite common in marine fish fed trash fish or a high-fat (20%) pellets (Fig. 12). Normal fish (freshwater and coastal marine fish species) have dark red to brown livers probably due to the high metabolic activity of the organ requiring significant blood flow. However, deep-sea fish use fat tissue for buoyancy and so have livers that are normally pale and fatty (Fig. 13). In starved fish, the liver that acts as a storage organ for fat and glycogen can be atrophied. The consistency of the liver should be firm through a fairly thin 'capsule'. Fish livers have lobes but they are less distinctive than in mammals. The gall bladder is a sac containing greenish-yellowish bile fluid, and its shape may be very elongated or pear-like. Normal bile is a clear, green fluid but diseased bile can be thick like a white exudate and this can be an indication of parasitism, e.g., myxosporidiosis. Myxosporean gall bladder infections (particularly with large numbers of trophozoites or spores) result in a reddened inflamed or swollen (obstructed) gall bladder (Meglitsch, 1959). The bile duct(s) are visible and the gall bladder can sit adhered to a fossa of the liver. There are two broad types of liver architecture in fish: a true liver with no pancreatic tissue and a hepatopancreas with islets of pancreas within the liver. The pancreas is not visible grossly as in mammals. It is a dispersed organ varying in location.

Figure 11a. Normal deep red liver of goldfish (*Carassius auratus*): Liver (L).


Figure 11b. Normal looking liver is reddish brown; Russell's Snapper (*Lutjanus russelli*).

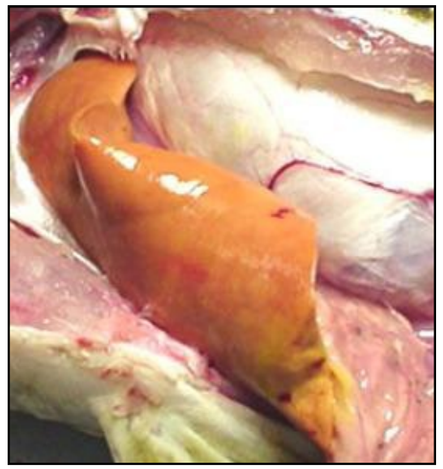


Figure 12. Yellow fatty liver of green grouper (*Epinephelus coioides*).

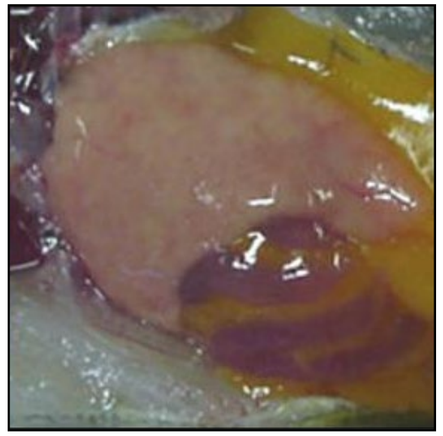


Figure 13. Pale fatty liver of Russell's snapper (*Lutjanus russelli*).

Swim Bladder

All fish swim bladders have an initial connection to the digestive tract formed as out pouches of the oesophagus, and in some fish, this connection remains patent throughout life. Fish are either physoclists (closed swim bladder pneumatic duct) or physostomes (open duct). This differentiates how inflation or deflation of the swim bladder occurs.

Physostome swim bladders allow for more rapid deflation and inflation. Depending on the species, gas may fill the bladder through the pneumatic duct connecting the swim bladder to the oesophagus and as the fish gulp air from the air-water interphase. The patency of this connecting tube may be compromised in fish with swim-bladder hypo- or hyperinflation.

Closed swim bladders (physoclists) are served by a rete mirabile or gas gland (Fig. 14). The gas is regulated by absorption or release from the blood via a capillary network in the swim-bladder wall. The inflation response is slower and is not usually found in river fish. Some physoclist fish have the swim bladder divided by a diaphragm into two compartments. The cranial part is for gas filling and the caudal part for gas release.

The very obvious swim bladder is a whitish air-filled sac situated in the dorsal section of the abdomen up against the spine of the fish. The swim bladder is thin in goldfish (Fig. 15), but quite thick and tough in marine grouper (Fig. 16). Several functions have been attributed to it, the main one being to assist the animal in maintaining depth or buoyancy in the water. The swim bladder in some species also has auditory and pressure reception functions. Roberts (2001) indicated that the gas composition of the swim bladder in physoclists (closed) are not necessarily atmospheric and in physotomes (open) that have no access to the air-water interphase, oxygen, nitrogen and carbon dioxide at concentrations different to atmospheric ratios can occur. For example, in physotomes such as cyprinids, the swim bladder contains pure nitrogen while carbon dioxide occurs mainly in physoclists. Fish with access to the air-water interphase and those with an open pneumatic duct may have gas in the swim bladder approximating that of the atmosphere. There may be a higher proportion of oxygen in deep-sea fish with a gas gland.

In the Superorder Ostariophysi, e.g., *Danio* spp. a Weberian apparatus joins the bones of several vertebrae to the swim bladder and inner ear, which allows the swim bladder to be part of the auditory system.

Figure 14. Swim bladder – rete mirabile or gas gland (arrow) with capillaries in grouper (*Epinephelus* sp.).

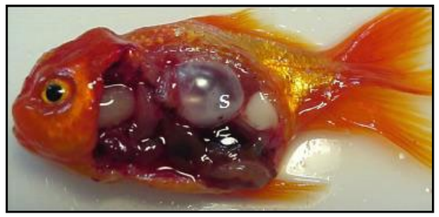


Figure 15. Swim bladder (S) – goldfish (*Carassius auratus*).

Figure 16. Swim bladder (S) – brown spotted grouper (*Epinephelus areolatus*).

Kidney

Fish have kidney tissue, which is not discrete as in mammals. The fish kidneys are dark red to deep brown organs located along the ventral part of the spine in a retroperitoneal position. In some species, there is a distinct cranial and caudal portion. The cranial kidney or head kidney is made up of haematopoietic tissue while the caudal portion has the excretory glomeruli and tubules, together with a variable proportion of haematopoietic tissue. The kidney is normally of a soft texture and contains a considerable amount of blood. However it should not be swollen beyond the retroperitoneal space, nor should it be jelly-like or watery with blood when cut – which may indicate necrosis.

The kidney is one of the vital sites for infectious pathogens to localise, with a relatively high blood content and is the most easily sampled in a sterile manner of such sites. Hence kidney samples are routinely taken to rule out bacterial or viral disease.

Figure 17. Kidney (K) of giant grouper (*Epinephelus lanceolatus*) along the ventral aspect of the spine.

Spleen

The spleen is an important organ in terms of immune and haematopoietic functions in fish. It is a dark red, flat to semi-oval-shaped or elongated organ found behind the stomach or the intestinal loops. The size and shape varies with the species and age of fish. It has a thin capsule and on cut surface should have a slightly spongy consistency. The spleen is normally another organ that may be cultured when suspecting or ruling out systemic infections, in fish of sufficient size. Some blood should ooze from the organ when cut, but it should not be swollen into a ball-like organ or have very rounded edges in healthy fish.

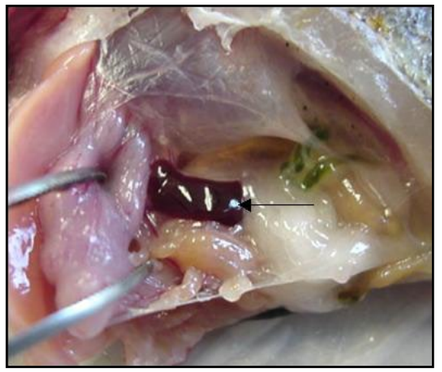


Figure 18. Spleen (arrow) of brown spotted grouper (*Epinephelus areolatus*).

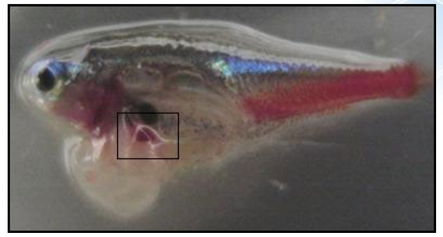


Figure 19. Spleen of neon tetra (*Paracheirodon innesi*).

Thymus

The thymus of fish is quite prominent, appearing similar to head kidney tissue being dark brown to reddish, but located in the dorsal aspect of branchial cavity at the dorsal commissure of the operculum, not in a retroperitoneal position. Immunologically competent fish should have a well-developed thymus. The function of the thymus is to produce differentiated lymphocytes and it should not be overlooked in gross or histopathological examination as it can indicate the immune competence of the fish.

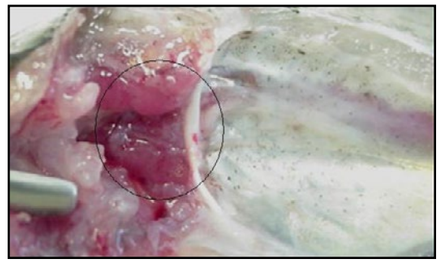


Figure 20. Thymus of green grouper (*Epinephelus coioides*).

Lateral Lines

These special sense organs are actually canals punctuated with pores along its length to give an appearance of a line running alongside the flanks of the fish and extending into a complex network of canals over the lateral surfaces of the head. It can be a common site for parasitic copepods (*Philichthyidae*) or other protozoal parasites. Water from the external environment flows through these pores and any disturbances are detected by sensory receptors, which connect to the central nervous system of the fish to elicit an avoidance response – the fish swims away from the potential predator.

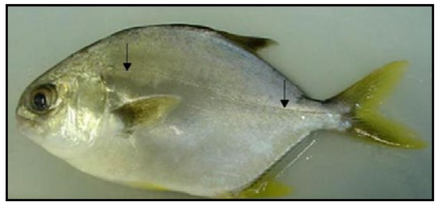


Figure 21. Lateral line (arrows) of a pompano (*Trachinotus blochii*).

Figure 22. Lateral line (arrows) with visible pores in a koi carp (*Cyprinus carpio*).

Brain and Spinal Cord

The fish brain has very obvious optic lobes which reflect the acuity of the visual function. These are usually the first parts that are visible when opening up the cranium. Ventrally there is a distinct blood sinus which may be mistaken for haemorrhage. The cerebellum tucks behind the optic lobes and is relatively small. The olfactory lobes sit in the telencephalon (forebrain) cranial to the two optic lobes. The fish spinal cord begins as the brain stem (medulla oblongata) and extends caudally in the spinal canal to end as a urophysis (which is an endocrine organ).

Eye

Fish have no eyelids but in eels there is a translucent membrane that protects the cornea. The fish eye has a spherical lens to support underwater accommodation of light and vision. The cornea is relatively thick, and there is an anterior and a posterior chamber with both aqueous and vitreous humour. The pupil dilates or constricts according to light intensity. Oculomotor muscles attach the globe to the orbit. The optic nerve connects the retina to the central nervous system. There is a highly vascular choroid organ in the back of the sclera of the globe.

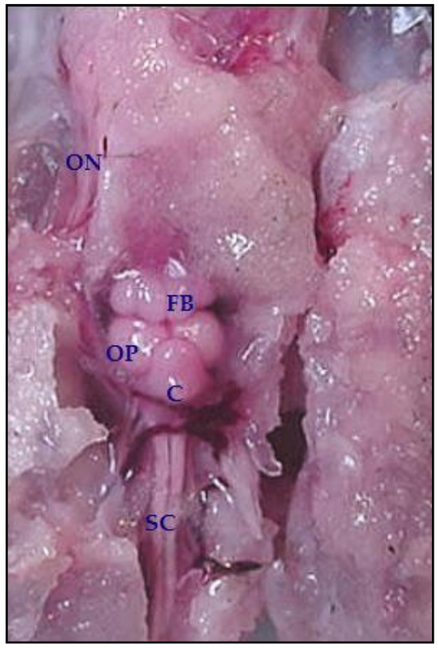


Figure 23. Brain and spinal cord of giant grouper (*Epinephelus lanceolatus*): forebrain (FB), optic lobes (OP), cerebellum (C), brain stem leading to spinal cord (SC) and Optic nerve (ON).

The Labyrinth

The lateral line connects to the labyrinth – which is the balance and auditory organ of fish. Damage to the lateral line by parasites can lead to imbalance of swimming in the affected fish. The labyrinth contains, on each side, three calcareous nodules – the otoliths. The largest otolith is used to age fish.

Gonads

The gonads of fish are only obvious during periods of reproductive activity and in mature fish. Some species have male organs to start with and then convert to female organs later in life, e.g., in barramundi (*Lates calcarifer).* This is termed asynchronous hermaphroditism. Testes are usually white (sometimes similar to fat tissue) but will have a milky appearance and be in the shape of paired and elongated lobes (Fig. 24). Testes are generally triangular in cross-section while ovaries are round. They are located adjacent to the swim bladder. Ovaries will have obvious eggs of varied size and colour. Immature eggs may be a pale grey colour (Fig. 25). The more mature ovaries will have orange-yellow eggs (oocytes) and this may occupy a large volume in the abdomen (Fig. 26). Bulging of the abdomen is common in female fish ready to spawn eggs.

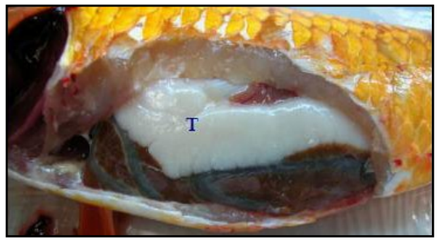


Figure 24. Koi carp (*Cyprinus carpio*): testes (T).

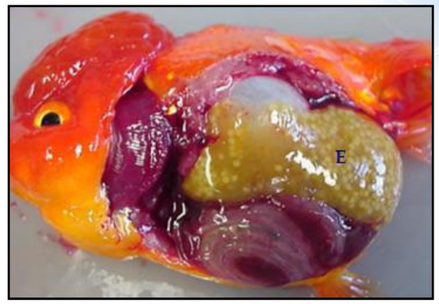


Figure 25. Goldfish (*Carassius auratus*): maturing eggs (E).

Figure 26. Neon tetra (*Paracheirodon innesi*): matured eggs (E).

Fish Physiology

Respiration

Fish respire through their gills. There is a vast surface area for gaseous exchange due to the design of the primary and secondary filaments and since the availability of oxygen in the water is lower than in the atmosphere, fish have a very efficient means of extracting oxygen through the counter-current circulation of the gills. This involves the flow of water over the gills being opposite to the flow of blood in the gill capillaries lending to maximum rates of gaseous exchange. Carbon dioxide is released from the blood haemoglobin while oxygen is adsorbed onto haemoglobin – a process that is regulated by blood pH (the Bohr and Root effects). These effects produce a decreased oxygen affinity when blood pH is reduced due to the build-up of carbon dioxide. The buccal ventilation rate of fish is determined by the chemoreceptors in the gills and pseudobranch which responds to blood PO_2 and PCO_2 levels. Ventilation rate is increased with declining PO_2 and rising PCO_2 levels. Clinically fish that are in hypoxic conditions come to the surface of the water or near the area of relatively higher dissolved oxygen levels and exhibit rapid gill or opercular movements. The oxygen requirements of fish are increased in the following situations:

- after feeding when additional energy is required to digest food.
- in elevated water temperatures when basal metabolism is increased, e.g., doubling for each 10ºC in the range of 20-30ºC for sockeye salmon (Roberts, 2001).

In such situations if there is inadequate dissolved oxygen (DO) or if gill function is impaired then hypoxia may occur leading to fish mortality. Conditions where DO may be reduced include:

- high water temperatures that depress oxygen solubility in water.
- bottom layers of water near anoxic sediments.
- reduced water flow rate.
- elevated salinities.
- excessive organic materials and nutrients in the water that have a high biological oxygen demand (BOD).

Fish species will have DO preferences where they are physiologically comfortable. For example, salmonids need DO at or greater than 5 ppm, while grouper and goldfish may tolerate levels below 5 ppm. But the absolute minimum DO for most fish species is 2-3 ppm. Below this, fish die as the energy required to extract the limited oxygen exceeds that derived from respiration. In general a DO of 5 ppm is recommended for aquaculture. When considering DO levels, the integrity of the gill function, presence of pathogens and the oxygen demand of the fish will influence whether hypoxia is an issue even if DO levels are at 5 ppm.

Osmoregulation

Two environments, freshwater or marine water, determine the direction of osmoregulation that fish require. In freshwater, water tends to flow from the external environment into the internal tissues of the fish. In saltwater, fish tend to dehydrate. Therefore osmoregulation being aimed at maintaining fluid and electrolyte constancy in the fish will be opposite between freshwater and marine fish. For brackish water fish or fish that transfer from a freshwater to a marine water environment, the osmoregulatory capabilities actually change in a process called smoltification for salmonid fish.

Freshwater fish need to conserve body salts and excrete lots of water by producing very dilute urine. The gills and kidney are involved but the integrity of the skin is very important to maintain an osmotic barrier. In the gills, chloride ions enter while bicarbonate ions from carbon dioxide exit; active sodium uptake can occur while in neutral or alkaline pH water, but is inhibited at low pH. The hydrogen ions exit which drives a net input of NaCl. In the kidney, salts are reabsorbed from the renal filtrate and excess water is excreted.

Marine fish need to conserve water and excrete excess salts. In the gills, chloride cells actively excrete sodium and chloride ions. The kidneys excrete magnesium and sulphate ions producing a very concentrated urine. Reduced numbers of glomeruli or lack of glomeruli in some species minimise filtration and hence water loss. At the same time marine fish drink in copious amounts of seawater to balance the water intake required.

Failure to osmoregulate leads to death. This occurs with damage to the gills, skin or kidney. Loss of the protective barrier of skin and gill injury, which impairs the electrolyte exchange mechanisms, are perhaps the most important insults that compromise effective osmoregulation.

Cardiovascular Function

The fish erythrocyte is nucleated, elongate-ovoid in shape, about 10 µm long and contains haemoglobin, giving it a bright red colour when oxygenated. Blood volume is about 2-4% bodyweight and lymph volume is 4-5 times blood volume (Ferguson, 1989). Erythrocyte production takes place mainly in the head kidney and spleen.

The fish heart pumps blood from the venous vasculature through the gills for oxygenation without a return to the heart, but directly to the arterial vasculature via the dorsal aorta. Cardiac contraction is stimulated by pacemaker tissue in the sinus venosus (Roberts, 2001). The sinoatrial, atrioventricular and ventriculobulbar valves prevent retrograde flow of blood in the heart. Perfusion of the gills may be dramatically increased through recruitment of unperfused gill lamellae as metabolic oxygen demand increases. This is regulated by adrenalin/noradrenalin β receptors. Cardiac output increases mainly by increased stroke volume and also by increased heart rate (Roberts, 2001).

Since the fish circulatory system is relatively low in pressure (not having to contend with gravity effects), this is reflected in the structure of the vasculature. The dorsal aorta wall is thinner and valves are few in the veins. However special design features to maintain adequate blood flow include an elastic ligament in the dorsal aorta, and a renal portal venous drainage of the tail region.

Immune Function

The first line of defence for fish is the skin and its mucus. The gills also have incorporated into its function, protective mechanisms such as mucus and cellular responses. Since the fish is intimately in contact with its environment – the water, which also is the medium that carries a multitude of potential disease organisms – the integrity of the integumentary barrier is vital to defences against pathogenic invasion. For example, if the skin has been slightly abraded by other fish in a densely stocked culture tank, then it favours the colonisation of opportunistic bacteria, which may progress to infection and septicaemia. Parasites and bacteria often feed on the skin and gill surfaces; however the continuous production and shedding of mucus which contains a variety of inhibitory enzymes (lysozyme, precipitins, agglutinins and transferrin) ensures that pathogens are effectively neutralised and removed from the fish. In the event of a bacterial infection or parasitic infestation, excess mucus is produced to protect the fish from further damage. Also the epithelial cell layers of the gills and epidermis often proliferate (hyperplasia) in an attempt to maintain integumentary integrity. However if the pathogenic pressure increases or continues or the fish is chronically stressed, the first line of defences will be breached and systemic disease occurs.

Beyond the integumentary defences, fish have both cell-mediated and serum-mediated immunity. Cells involved in cell-mediated defences are the macrophage, lymphocyte, neutrophils (heterophils), sometimes eosinophils, possibly basophils and the fixed tissue mast cell equivalent that is commonly seen as an eosinophilic granular cell. Phagocytic cells are the macrophages (derived from the circulating monocyte) and the neutrophils. They engulf and neutralise both pathogens (bacteria and virus) and particulate matter (metabolic by-products and foreign material). Eosinophils are usually involved in parasitic defences but also can phagocytose antigen/antibody complexes. Basophils are rare and their functions are as yet unclear in fish. Mast cells and their precursors can be said to occur and these include the fixed eosinophilic granular cells that are often very common.

Fish are equipped with specific and non-specific immune responses. Antibody production subsequent to antigen exposure occurs when the fish is of sufficient maturity. This is important when considering the best age or size to vaccinate fish so as to obtain the best protection from the disease of concern. Specialised lymphoid cells produced in the head kidney, spleen and thymus deliver specific immunity. Therefore disease conditions, which impinge on these organs, can cause immunosuppression. Antibody producing cells include the B and T lymphocytes, and macrophages. Antigens are processed primarily in the reticuloendothelial tissue of the head kidney and spleen. Often the presence of melanomacrophage centres (MMCs) indicates active antigen processing.

Conditions that affect the function of fish immunity are:

- water temperature – lower temperatures in the physiological range of the species will depress the immune response and wound healing.
- nutrition – malnutrition (e.g., proteins, essential fatty acids and vitamins, e.g., C) depresses the immune response.
- chronic stress – suboptimal water chemistry conditions, highly organic environments, crowding and poor husbandry depress the immune response.
- chemical treatments – some drugs/chemicals are potentially immunosuppressive. These include oxytetracycline – a commonly used drug in aquaculture.

Therefore vaccination programmes must manage these factors to provide the best protection to the fish stocks that are vaccinated.

Endocrine Function

Fish have hormone functions just like vertebrates. Endocrine control of physiological and metabolic processes is well documented. Table 1 summarises the major hormone groups in fish. From a clinical viewpoint an understanding of the following endocrine processes is important:

- osmoregulation (prolactin, cortisol, Corpuscles of Stannius, vasotocin, arginine).
- stress response (cortisol, adrenalin, noradrenalin).
- reproduction (pituitary, oestrogen, androgen, oxytocin, melatonin).
- digestion and nutrient metabolism (insulin, glucagon, thyroid hormones).
- growth (growth hormone, somatostatin).
- cardiovascular regulation (angiotensin, urotensin).

Dissection of fish tissues rarely allows visualisation of hormone producing tissues. Histology may reveal a number of endocrine tissues, e.g., the islet of Langerhans (endocrine pancreas), the interrenal gland (cortisol), the gonads and the hypothalamus-pituitary.

As fish are greatly influenced by environmental factors such as water chemistry, it is necessary to understand these interactions in order to appreciate the finely tuned endocrine mechanisms that maintain an optimum internal environment in the face of often rapidly changing external conditions. Failure to respond swiftly to external variations leads to morbidity and mortality in fish. Similarly, in husbandry, if external conditions are changed too rapidly, the hormonal mechanisms are not able to make metabolic adjustments at the same rate, and this results in the stress or death of the animal.

As an example, the control of osmoregulation is critical to the survival of fish in the face of fluctuating water chemistry. Cortisol assists in the maintenance of fluid volume particularly in marine fish as dehydration tends to occur. Therefore a decrease in salinity would trigger a reduction in cortisol secretion to maintain osmotic pressure in the internal tissues. A rise in salinity would require sodium extrusion and this is mediated through the activation of chloride cells by cortisol.

Another example of endocrine regulation is that of growth. The hormones involved are thyroxine/triiodothyronine and growth hormone, and these are in turn regulated by the pituitary-hypothalamus axis. Growth hormone stimulates appetite and protein production while thyroid hormones regulate the metabolic rate, which is also influenced by environmental temperature. Somatostatin on the other hand controls growth through its action of inhibiting the release of growth hormone.

Hormonal regulation of nutrient metabolism is particularly relevant for aquaculture in terms of nutrient composition and the prevention of nutritional disorders, which is commonly manifested as poor growth or poor food conversion efficiency. Insulin and glucagon are involved in regulating carbohydrate metabolism. An example of diabetes mellitus in fish is Sekoke disease of Japanese carp feed silkworm pupae due to the loss of functioning pancreatic β cells.

Table 1. Functions of fish hormones

Hormone	Functions	Sites of Action/Regulation
Prolactin	Osmoregulation - maintains blood sodium and calcium levels. Promotes melanin production. Maintains mucus production.	Gills and kidney. Melanophores of skin. Epidermal mucus cells.
Growth hormone	Stimulation of appetite and protein anabolic effects.	Islets of Langerhans produces somatostatin, which inhibits the release of growth hormone.
Melanophore regulating hormone	Concentrates melanin granules in the centre of the melanophore leading to lightening of fish colour.	Stimulates melanophores in the skin. Works in conjunction with neural inputs.
Arginine vasotocin	Osmoregulation	Gills
Oxytocin	Reproduction	Gonads
Thyroxine and triiodothyronine	Growth regulation via carbohydrate metabolism. Pigmentation – increases guanine levels in scales, which causes a silver colour. Smoltification in salmonids.	TSH feedback loop via the hypothalamus – pituitary axis.
Insulin	Pancreatic beta cells produce insulin, which reduces blood glucose and amino acid levels.	Liver
Glucagon	Pancreatic alpha cells produce glucagon, which raises blood glucose.	Liver
Somatostatin	Produced by pancreatic delta cells, which inhibits the release of growth hormone.	Pituitary
Cortisol	Causes water retention in freshwater fish and dehydration in marine fish. Stimulates gluconeogenesis, conversion of glycogen to glucose. Causes neutrophilia and lymphopenia which can cause reduced immune functioning and disease in chronically stressed fish.	Acts on the gill 'sodium pump', kidney and gastrointestinal tract. Liver and muscle.
Adrenalin and noradrenalin	Secreted by the chromaffin cells in response to stimuli of 'flight, fright and fight'. Increases heart rate, causes peripheral vasoconstriction, vasodilation of coronary vessels and increased glucose supply from glycogen stores.	Heart, blood vessels and liver.
Corpuscles of Stannius hormones	Calcium regulation in fish includes hypocalcaemia hormones, e.g., stanniocalcin (STC) and calcitonin (CT), and hypercalcemia hormones, e.g., prolactin (PRL) , somatolactin (SL) and a parathyroid hormone-related protein (PTHrP). The corpuscles of Stannius produces STC. The pituitary gland produces PRL, SL and PTHrP. Fish do not possess discrete parathyroid glands, nor produce parathyroid hormone (PTH). Calcium regulation is mediated by changes in water osmolality and sodium chloride concentration changes rather than calcium ion levels (Evans and Claiborne, 2006)[1]. Development of gill chloride cells.	Gills. Feedback from changes in the ionic composition of water.
Calcitonin	The ultimobranchial gland produces calcitonin, which decreases blood calcium.	Gills.
Melatonin	Produced by the pineal gland and retina, and controls reproduction whilst also being involved in smoltification.	Gonads.

Renin-angioten-sin	Renin is produced in juxtaglomerular cells, which are modified smooth muscle cells of the afferent glomerular arterioles of the kidney. Renin triggers the conversion of angiotensogen to angioten-sin I and II, which increases blood pressure by vasoconstriction and sodium retention.	Blood vessels, gills and kidney
Urotensins	Produced by the urophysis. Urotensin I (U-I) acts directly on the interrenal gland to stimulate cortisol secretion in some seawa-ter-adapted teleosts; it also stimulates Cl^- active transport in goby by the opercular epithelium, and inhibits water and NaCl transport across the anterior intestine of freshwater tilapia but not marine fish. Urotensin II (U-II) also acts on interrenal tissue to cause cortisol secretion, reduces Cl^- excretion across opercular skin and promotes Na^+ absorption in the urinary bladder of goby. U-II also stimulates absorption of water and NaCl in the intestinal epithelium of tilapia (Evans and Claiborne, 2006)[2].	Interrenal gland, opercular epithelium and intestine.

Reproductive Function

There are numerous reproductive methods in fish. Most species have separate male and female fish. Others are hermaphrodites, i.e. having both male and female gonads in one fish at the same time such as occurs in sea bass and the stickle back. But within the hermaphrodites there are species, which are initially male but develop female gonads later in life. These are called protandrous hermaphrodites. Examples include seabream, porgy and clownfish. Another group are the protogynous hermaphrodites, which are first females and then become males later, as in swamp eels. Other hermaphrodites are unisexual in which only female juveniles are produced from female brood fish.

Fish are cued to spawn by the appropriate environmental conditions through the pituitary-pineal gland – usually due to increasing day length, which corresponds to spring/summer when food sources are more abundant. Gonadotrophins and reproductive hormones such as oestrogen and androgens are intricately involved in the maturation and spawning of gametes, similar to the system in mammalian vertebrates. This allows a degree of control over the timing of spawning through the use of synthetic gonadotrophic hormones. In species like the tilapia, where the male animal is desired because of better growth and to avoid rapid reproduction of next generations in a pond (leading to poor growth and uneven sizes of fish), female brood fish are treated with an androgen (testosterone) to produce an all-male population.

Once spawned, fish eggs may be artificially fertilised by the sperm (milt) stripped from the males. Egg development is dependent on a water hardening process as in salmonids, which prevents multiple sperm penetration, and also allows the fertilised egg to withstand damage from the environment. Eggs generally require a low-light environment and appropriate water temperatures for development and hatching into fry. Salmon eggs naturally are laid in gravel and hence do not require direct lighting during incubation, and a higher percentage of Atlantic halibut (*Hippoglossus hippoglossus*) eggs will survive to hatching if incubated in the dark or low light levels (Stickney, 2009). The higher the temperature within the optima, the faster the time to hatch. Oxygen, water flow and fungus are critical issues regarding proper egg development. Once hatched the fry rely on the yolk sac (in those species that have a large yolk sac, the time to swim up is longer than those with a small yolk sac) for early nutrition. The fry depend on proper inflation of the swim bladder. Failure of this inflation can be due to the presence of an oily water/air interface or other inhibitory stresses. This can result in the inability of the fry to eat properly food items such as *Artemia* and *Branchionus.*

Fish species that bear live young do not lay eggs and mouth brooders take care of their hatched fry for various periods in the mouth cavity. Usually the male fish performs this task and does not feed during the mouth brooding period.

Digestive Function

The digestive tracts of fish are designed to capture, masticate, swallow, digest and assimilate food organisms, plants and particles. The diversity of feeding habits, food types and digestive function is an important fact to note. Fish are able to detect food in the water through sensory acuity via the taste buds in the oral epithelium as well as specialised barbs located outside the mouth on the lower lips. Once food is captured, it is manipulated by the tongue and often swallowed whole. The stomach in most fish (there are fish species that do not have a stomach) can distend to a large size to hold food and it is here that digestion commences because true enzyme secreting salivary glands are absent in fish, though lubrication via mucus cells does occur. Hydrochloric acid and pepsin secretions from the glands of the stomach enable protein digestion. Other non-proteolytic enzymes can be present in the stomach. These include amylase, lipase, esterases, chitinase, hyaluronidase and cellulase for digesting crustacean chitin and carbohydrates. Pancreatic secretions such as bicarbonates empty into the proximal intestine to neutralise the hydrochloric acid. Pancreatic enzymes that facilitate protein digestion include trypsin, chymotrypsin, elastase and carboxypeptidases. Pancreatic lipases digest fats while pancreatic amylases and chitinase promote carbohydrate and chitin digestion. Bile secretions from the liver and gall bladder contain bile salts (detergent action) involved in the emulsification of lipids. Intestinal enzymes secreted from the mucosa include polynucleotidases which breakdown nucleic acids, peptidases that breakdown protein peptides and lecithinases that breakdown phospholipids. Gut bacterial microflora may assist in the digestion of cellulose and chitin for some species.

The activity of digestion has a direct energy and oxygen cost to fish called the 'specific dynamic action'. In practice, this means that feeding fish under marginally dissolved oxygen conditions can kill fish after feeding due to an increase in fish oxygen demand that strips the available oxygen from the water. Hormonal control of the digestive tract is achieved through the interplay of the parasympathetic and sympathetic nervous systems. Gastric acid secretion is triggered by stomach dilation. Pancreatic exocrine secretions are stimulated by the passage of acid gastric secretions into the intestine. Pancreatic hormones such as insulin and glucagon are important in the digestion, absorption and regulation of carbohydrates. Bile emptying from the gall bladder is controlled by vagal nerve function. When fish have not eaten for a while, the gall bladder is often distended with bile. Gut motility is influenced by the water temperature, gut length and food types. For example, fish with long intestines have gut emptying times of 6-10 hours at 8 ºC (e.g., mullet) while fish with short intestines have prolonged emptying times of 49 hours at 8 ºC (e.g., blennies) (Munday, 1990).

Excretion of Metabolic Wastes

Fish generate nitrogenous wastes in the form of ammonia which accounts for 85-90% of total waste nitrogen. Ammonia is excreted mainly through the gills by simple diffusion. Ammonium ions (NH^{4+}) are converted to NH_3 at the gills and released in exchange for Na^+ (Munday, 1990). A smaller proportion of nitrogenous waste is excreted as creatine via the kidneys. The main issue with excreted ammonia waste is that it is toxic to fish itself because the waste remains in the immediate environment of the fish unless it is removed by biofiltration processes of nitrification or clean water is exchanged into the culture environment to dilute away the toxin.

Being in the aquatic environment, fish can be exposed to a myriad of dissolved compounds ranging from heavy metals to man-made pesticides and pollutants. Fish kills commonly occur, giving an indication of the declining health of the aquatic ecosystem. To a certain extent, a fish is able to detoxify exogenous compounds through the biochemical conjugation processes in the liver and excretion via the kidney. Much of the detoxification of toxicants occurs in the liver, but also in the gills and kidney which is mediated by the cytochrome P450 enzyme (mixed function oxidases) systems (Di Giulio and Hinton, 2008). However, bioaccumulation and synergistic effects of pollutants can easily overwhelm the capacity of fish to detoxify and maintain homeostasis. This will lead to degenerative pathologies in the liver, gills and kidney.

Nervous System Function

Fish have a highly functional nervous system similar to mammalian vertebrates, comprising voluntary and autonomic systems. See the following notes on the sensory functions of teleost fish. Motor control is effected by the brain, spinal cord and peripheral nerves. Damage to these structures, e.g., in viral infections, manifest as aberrations in swimming coordination and swim bladder control leading to whirling and circling abnormalities. Colour of skin is also regulated by nervous input into skin chromatophores. A stressed or sick fish will often be darker in colour due to the expansion of chromatophores with the pigment granules distributed in the dendritic processes of the cell. Much more intense darkening that is sharply demarcated occurs when there is complete loss of nervous control, as may occur when a section of the spinal vertebral column is parasitised by the myxozoan *Myxobolus cerebralis* resulting in cranial or spinal cartilage deformity. Spinal fractures can result from the deforming lesion, also causing a blackened tail. Affected fish have a dark coloured tail end due to the loss of innervation to the chromatophores distal to the spinal deformity lesion. Metabolic functions of respiration, osmoregulation, digestion, and cardiac contraction are all under various degrees of neurological control in response to environmental changes.

Vision

Fish often live in low light conditions and their eyes are designed to maximise the available light for adequate vision through the complex structures of the cornea and retina. The cornea has a refractive index commensurate with that of water. As fish do not have eyelids, the cornea is quite tough, being made up of five layers (epidermis, Bowman's membrane, substantia propria, internal basement membrane and the endothelium). The retina has rod and cone receptors to trap light and a pigment layer with rhodopsins enable colour vision for species in shallow water. The spherical shape of the lens enables fish to have a wide angle of vision. The extensive capillary network behind it called the choroid enhances oxygenation of the retina. Diseases with ocular manifestations include exophthalmia and corneal ulcerations (infections), blindness (viral encephalopathy and retinopathy) and cataracts (eye fluke, zinc deficiency). Cataracts are not specific to zinc deficiencies or mechanical disruption as with flukes. Other deficiencies such as histidine have been implicated. Secondary zinc deficiency due to poor absorption can occur with other nutritional imbalances and hence investigation may need to go beyond measuring zinc levels.

Olfaction

Fish have an acute sense of smell having the ability to detect very small concentrations of dissolved chemicals in water. This is a protective mechanism to enable fish to avoid areas of polluted water. It also is used by fish for the seeking of food sources through the detection of attractant compounds such as amino acids. Commercial feed formulations often include such attractants to improve the palatability of the feed. The olfactory receptors are found in the nostril chamber, which is perfused by water.

Lateral Line System (LLS)

The LLS is an important defence function of fish suited to the aquatic environment because it alerts fish to swim away from potential dangers, e.g., predators. It also enables proper schooling behaviour in some species that school, e.g., sardines. The LLS is made up of a canal in the skin of fish, which is punctuated along its length with holes in the scales, thus allowing the inflow and outflow of water. It allows pressure waves to be detected in the vicinity of the fish, which may be due to an approaching predator. Delicate cilia detect movements in the water column of the LLS canal, which sends nervous signals to the Central Nervous System (CNS), alerting the fish to take evasive action. Skin lesions and parasites can damage the LLS system leading to abnormal swim response behaviour of the affected fish. Similarly damage to the CNS can impair the functioning of the LLS.

Hearing

The hearing function of fish is supported by resonance of the swim bladder acting as an amplifier for fish. Sound detection serves to alert fish to predation danger and to enable the location of prey or food sources. This is similar to the function of the LLS. Hearing organs include a Weberian apparatus of bones connecting the swim bladder to the cranium as in carps, extensions of the swim bladder to the labyrinth as in herring and anchovies or capture of air bubbles in the oral cavity as in eels.

Stress Response

Cultured fish experience a wide range of stressors from their environment (Table 2) and may respond to changes in their environment in the following ways:

- change in metabolic rate and hence oxygen demand.
- utilisation of different partitioned energy reserves.
- adjustments in osmoregulatory, respiratory-circulatory and hormonal functions.

Each of these physiological processes aims to establish homeostasis or balance in fish so that survival in the midst of environmental change is maximised. Where situations occur that exceed the ability of fish to maintain homeostasis, fish become stressed and adverse physiological effects begin to occur (Fig. 27). These stress-induced effects include:

- reduced resistance to infections or decreased immune function.
- osmoregulatory and respiratory compromise.
- toxication due to decreased ability to neutralise biotic or abiotic toxins.
- organ degeneration leading to organ insufficiency.
- death from organ failure and/or secondary infections and disease.

Figure 27. Relationship between environmental stress and health of fish.

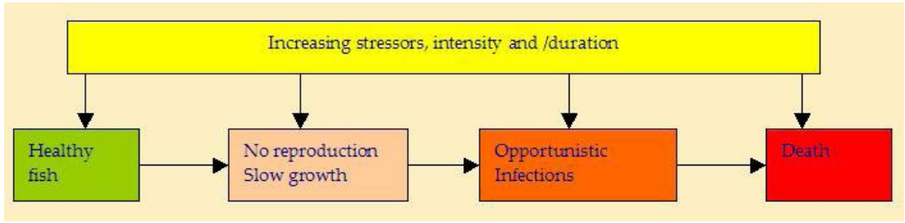


Table 2. Sources of stressors for cultured fish.

Stressor	Effects on fish
Reduced dissolved oxygen (DO)	Reduced oxidative energy production causing decreased cell and organ function. If severe, causes cell death and organ failure.
Increasing dissolved carbon dioxide	Reduced oxygen delivery to organs due to reduce affinity of haemoglobin for oxygen. Increased tissue acidity and acid-base imbalance with an adverse effect on osmoregulation at the gills.
Increasing ammonia in the culture water	Toxic effect on gill lamellar causing structural change (hyperplasia) resulting in reduced gill efficiencies and impacts on respiration, osmoregulation and excretion.
High stocking density	Increased production of metabolic and feed wastes, which pollutes the water. Increased biological oxygen demand due to high biomass and organic matter released in the water. Increased multiplication of pathogens and increased rate of spread due to close contact and increased risks of abrasions. All of these cause increased frequency and severity of disease outbreaks.
Inadequate biofiltration and water exchange	Build-up of metabolic waste compounds – damage to gills from toxicity, mortalities from either toxicity and/or secondary infections. Increased build-up of organic biofilm on surfaces, which favours the proliferation of pathogens.
Increasing water temperatures	Reduced solubility and availability of oxygen in the water. Increased fish metabolic rate and oxygen demand leads to relative oxygen insufficiency. Many pathogens proliferate better at high temperatures and attack fish tissues more readily. Increased fish mortalities either from hypoxia or primary infections.

Fish Histology

Gills

Understanding the normal histology of fish gills is very important for the pathologist to identify how the changes in the culture environment may adversely impact on the health of fish. Fish gills are very delicate tissues and unless the gills are properly preserved, many artifactual changes can occur which can confuse the pathologist and lead to an incorrect interpretation of what is observed in the gill section. Gills from a freshly killed fish need to be fixed within 2-3 minutes to minimise post-mortem artifacts. Remembering that what is seen on a gill section is a two-dimensional image of a three-dimensional structure will help the pathologist. Normal fish gills in proper 'right angle' section should display as in Fig. 28. Here we see that the secondary lamellae are evenly cut and form a full presentation from the base to the tips. Fig. 29a H&E shows a transversely cut gill section, which makes interpretation more difficult. TIP note: when examining the histology of gills, keep in mind the angle of sectioning. Hyperplastic artifacts can occur when the section is oblique to the plane of the gill surface (Fig. 29b). Make your interpretation based on adequately fixed gills and right angle sections of the gills. Gill artifacts include lamellar lifting, aneurysms and dishescence.

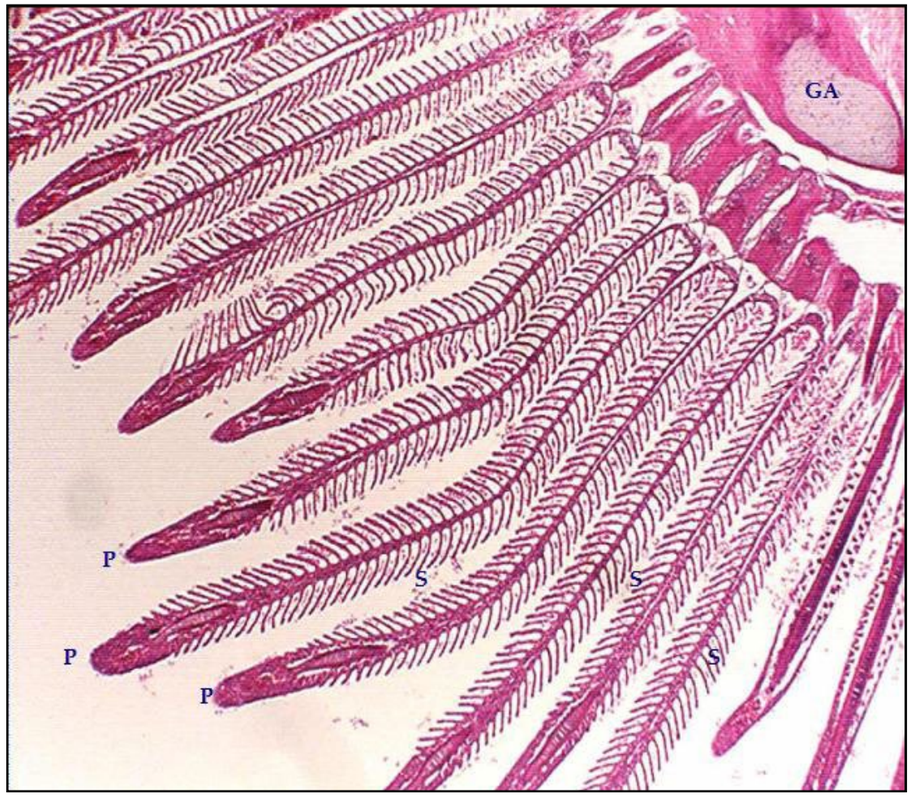


Figure 28. Fish gills stained with haematoxylin and eosin (H&E) showing: primary (P) and secondary (S) filaments. The gill arch (GA) with its cartilaginous support carries the gill filaments.

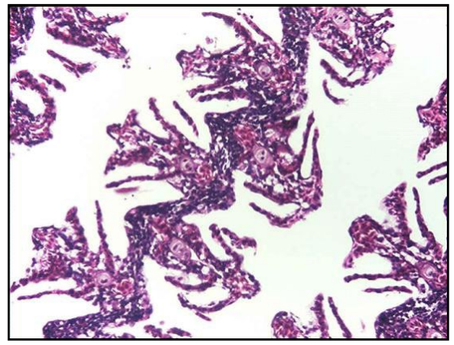


Figure 29a. Transverse gill sections.

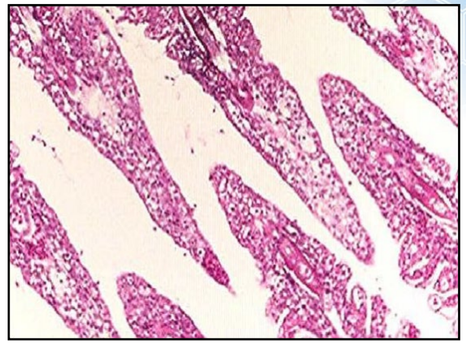


Figure 29b. Oblique gill sections (H&E).

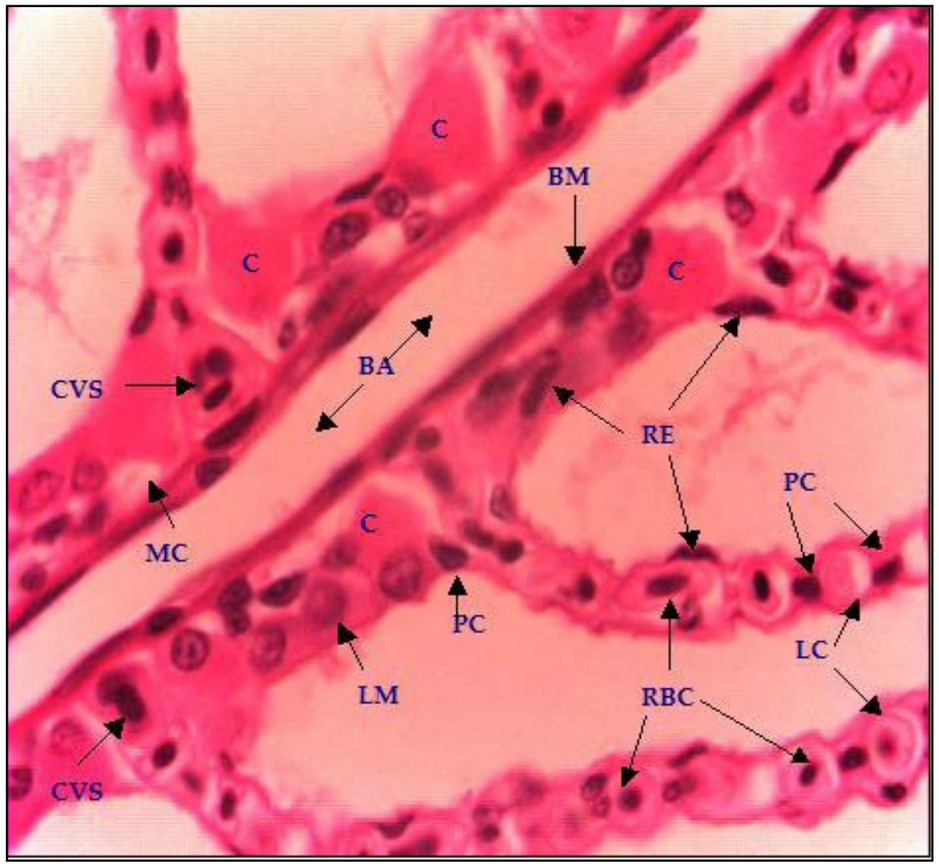


Figure 30. Marine grouper (*Epinephelus* sp.) gills (H&E): secondary lamellar epithelium and chloride cells (C), pillar cell (PC), lamellar capillary (LC), afferent/efferent branchial artery (BA), red blood cell (RBC), respiratory epithelial cell (RE), lamellar macrophage (LM), mucus cell (MC), basement membrane (BM), progenitor cell (PC) and central venous sinus (CVS).

The fish gill is designed to accommodate three separate functions – absorption of oxygen and removal of carbon dioxide, excretion of nitrogenous waste (ammonia), and osmoregulation. In the aquatic environment, these metabolic functions require:

1. adequate surface area for contact between the external environment (water) and the internal environment (blood).
2. minimal energy expenditure, hence a thin enough interphase.
3. protection to maintain the integrity of the internal host tissues.

To these ends, when as pathologists or veterinary clinicians we examine the histological structure of the fish gill, we can appreciate that the specific features are in place to support effective gill function. These features include:

The primary filaments (filaments) supported by the cartilagenous/bony gill arch. From these project the fan-like secondary filaments (lamellae) and together provide a very large surface area to volume ratio (Fig. 28). Sectioning of the gills shows how thin the epithelial membrane is across which gaseous, waste and solute exchange can occur, being only a flat single cell layer separating the external environment and the blood capillary (Fig. 30). As part of the defence mechanism, mucus cells can produce copious quantities of mucus which helps to remove attached parasites or pathogens away from contact with the delicate gill surface. They also reduce passive osmotic ion movement. With any chronic irritation, hyperplasia of the lamellar epithelial cells occurs to reduce the absorption of harmful substances or to ward off pathogens.

However, the downside of this is that the gills are highly susceptible to changes in the aquatic environment. For example, toxic substances can rapidly be absorbed through the gills and damage the internal environment of fish. Hyperplastic and mucus responses can increase the diffusion distance of the epithelial membrane which leads to reduced efficiency of gaseous, excretory and electrolyte exchange. Similarly, acute inflammtory infiltrates can disrupt the normal architecture of the gills with impairment of gill function.

Specialised cells include the chloride cells which sit at the base of the lamellae, and these provide active excretion of salts (NaCl) in marine and brackish water species, but are rare in freshwater fish. These have an eosinophilic cytoplasm (due to many mitochondria) and are intimately connected between the vascular space and the external environment (Fig. 30).

Gill repair occurs via the progenitor cells which migrate from the base of the lamellae towards the distal tip of the filament. Pillar cells which form the lamellar capillary with adjoining respiratory epithelial cells hold this delicate gill structure together. Blood cells are seen to meander their way through the lamellae moving unidirectionally from the afferent to the efferent arteriole.

Pseudobranch

This organ is lamellar in structure (Fig. 31), situated in the dorsal pharhyngeal area. It is well vascularised and has a good surface area for exchange. It looks like gill except that it is a hemibranch. Its role is to supply oxygen-rich blood to the eye via the choroid rete for the retina. As it is superficially located, it may be susceptible to disease processes that extend from the buccal or branchial tissues. Fish that do not have the pseudobranch, also do not have a choroid rete but have retinal specific blood vessels (Ferguson, 2006).

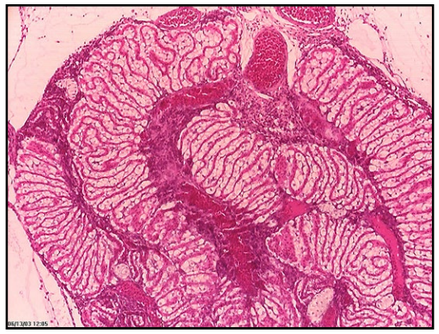


Figure 31. Goldfish (*Carassius auratus*) pseudobranch (H&E).

Skin

Fish skin is a very important organ in the aquatic environment. Its highly specialised structure reflects the unique challenges of living in water. Made up of layers, fish skin histologically is as follows (Fig. 32):

- The outermost layer is the mucus 'cuticle' which is secreted by mucus-goblet cells embedded in the epidermis. This mucus is the first line of defence against irritants and pathogens.
- The epidermis of fish consists of living, non-keratinised squamous cells from the basal to the top layers. This is important as it facilitates rapid repair of damaged skin. In some species, club cells occur which produce 'fright substances' to alert schooling fish to respond to predator threats.

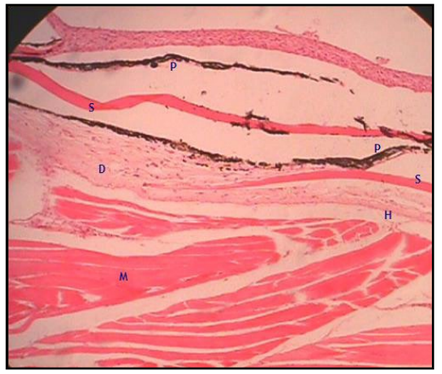


Figure 32. (H&E) Skin : pigment cell layer (P), dermis (D), hypodermis (H), muscle (M) and scale (S).

- The pigment cell layer lies beneath the epidermis and there are three main types of chromatophores: melanophores (black colour), xanthophores (orange/red colour) and iridophores (silvery colour). Colour change in fish occurs rapidly, under nerve control. For example, darkening in fish, which signals illness, is produced by the distribution of pigment granules moving outwards into the cell processes of the melanophores. This blocks light hitting the iridophores which contain reflective guanine plates.
- A scale is a calcified plate which gives some rigidity to the fish skin. Each sits in a scale pocket overlapping each other in a unidirection. Scales sit beneath the epidermis and portions of the scale embed into the dermis to maintain integrity.
- The dermis is a fairly thick collagenous layer which affords a good barrier against invading organisms.
- The hypodermis is a fairly loose layer of connective tissue where the blood vessels and inflammatory cells stand ready to respond to damage to the skin. Muscle layers connect to the skin via the hypodermis.
- It is routine also to note the lateral line canal which contains sensory neuromasts. Parasites may be in the canal, potentially causing neuromast damage (Fig. 33).

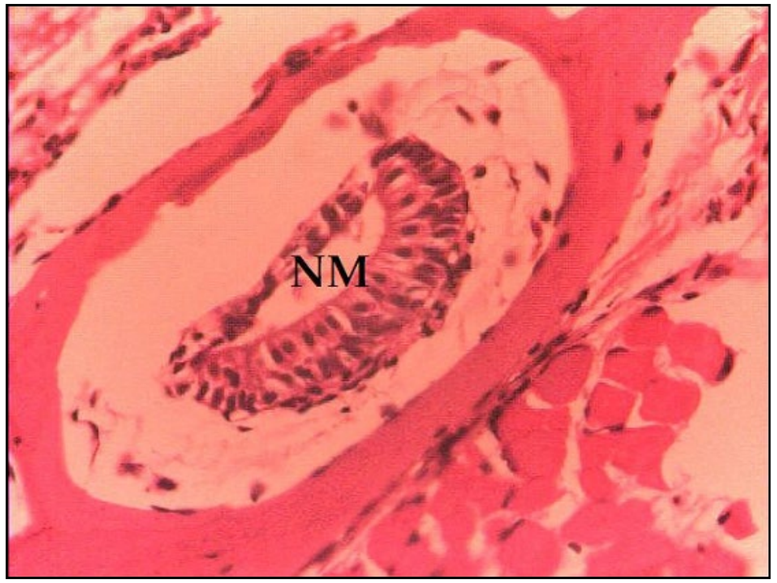


Figure 33. (H&E) Lateral line canal with cells of the neuromast (NM).

Kidney

Fish kidney is made up of haematopoeitic tissue and excretory tubules. The haematopoeitic tissue consists of erythropoeitic and lymphopoeitic blast cells. There is more haematopoeitic tissue in the cranial kidney and more excretory tubules in the caudal kidney although the distinction is not absolute, and varies between species. TIP note: for the pathologist, cutting in the middle portion of the fish kidney enables examination of both haematopoeitic and excretory elements of the fish kidney. When examining the haematopoetic tissue, it is important to note that the kidney acts as a filter for antigenic material and therefore the presence of inflammatory processes. The melanomacrophages often aggregate to form melanomacrophage centres (MMCs) which contain antigens or foreign particulates or parasites (Fig. 34). MMCs act as storage and processing sites for antigens, catabolic products or insoluble particulates. Melanin produced in the MMCs appear black, though other metabolic end products also contribute to the dark appearance. The melanin is involved in protecting host tissue from the oxidative effects of reactive free radicals released by macrophages to destroy pathogens. Many black MMCs can mean that the fish has been exposed to a degraded aquatic environment if there are no parasites or microbes involved in the MMCs. MMCs can also contain ceroid, lipofuscin and haemosiderin. Ceroid and lipofuscin come from lipid peroxidation. Haemosiderin comes from haemoglobin breakdown. TIP note: ceroid and lipofuscin is yellow-brown in H&E but special staining techniques are required to identify them. For example, haemosiderin is blue when stained with Perls Prussian. Lipofuscin is black when stained with Schmorl's and ceroid is red when stained with Ziehl-Neelsen. In general, increased numbers of large and black MMCs indicate that a fish has been sick and subjected to infectious and/or non-infectious stressors. The MMCs also indicate the level of catabolism or tissue damage sustained by the fish in situations such as malnutrition, starvation or physiological stressors, e.g., gonad resorption, or simply ageing.

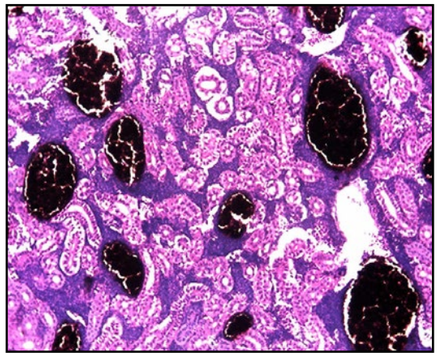


Figure 34. Pompano (*Trachinotus blochii*) kidney (H&E) with MMCs.

The fish glomerulus or nephron unit (Fig. 35) is more numerous in freshwater fish that have to excrete excess water to maintain osmoregulation. In marine fish glomeruli are few as these fish need to conserve water. The Bowman's space is an indicator of the excretory function of the kidney. Where there is dilatation (> 20% of the total glomerular diameter with species variation) (Fig. 36), it can indicate abnormal glomerular filtration leading to osmotic imbalance. However, chronic glomerulonephritis may cause occlusion of the Bowman's space due to thickening of the glomerular membrane or inflammatory infiltrates.

The fish renal tubules (Fig. 37) have cuboidal epithelium in the proximal segments and more columnar epithelium towards the distal collecting ducts. One feature to look out for is the presence of eosinophilic droplets in the cytoplasm of the renal epithelium which is a sign of degenerative change. Vacuolar change in the epithelium is also a sign of degeneration. In the absence of parasites or infection, these changes are possibly related to toxic insult or environmental stressors. TIP note: care is needed when interpreting renal necrosis as sloughing of epithelial cells into the lumen can be due to autolysis. If there is eosinophilic or vacuolar degeneration along with cell sloughing, then this would more likely be genuine tubular necrosis.

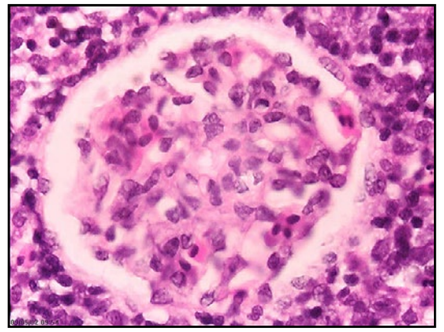


Figure 35. (H&E) Glomerulus.

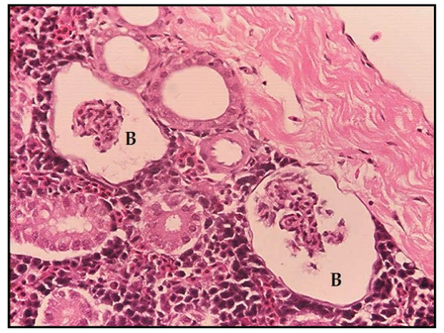


Figure 36. (H&E) Dilated Bowman's space (B) in glomeruli of pompano (*Trachinotus blochii*).

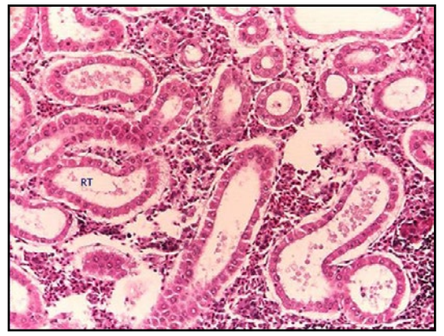


Figure 37. (H&E) Renal tubules (RT).

Liver

Liver histology in the normal fish is variable. This is particularly so when assessing the fat content and in knowing when to call it abnormal, especially in species where the liver is the major fat storage organ. (Fish with pyloric caeca often have both the pancreas and fat between the caecae.) Fish livers can store a large amount of fat and the fish is clinically healthy. However there is increased risk of lipid auto-oxidation if the liver is abnormally fatty. Oil red O-triethyl phosphate stains fat in frozen liver sections bright red (Fig. 40), while Sudan Black stains lipids black (Fig. 41). TIP note: if the liver is highly vacuolated with fat globules in the cytoplasm and a marginalised nucleus which is dark or condensed or pyknotic with the presence of deposits of eosinophilic droplets in the cytoplasm together with loss of an intact cell membrane, this is more likely to be fatty hepatocellular degeneration (Figs. 42-44). Normal hepatocytes appear tightly packed together, containing a centrally located nucleus with a distinct nucleolus, and cytoplasm containing variable amounts of granular material (which is glycogen). There should not be any significant amount of pigment in the hepatocytes except perhaps bile pigments. The liver section should have normal vascular and bile duct structures (Fig. 38) and be interspersed in the parenchyma maybe hepatopancreas in some fish species (Fig. 39). Other fish with 'true livers' have no pancreas in the liver as in trout. There are no distinct hepatic lobes or portal triads as in mammalian livers, although lobules may occur for example in *Lates calcarifer.* MMCs may occur if there is an overflow of MMC activity from the spleen or kidney.

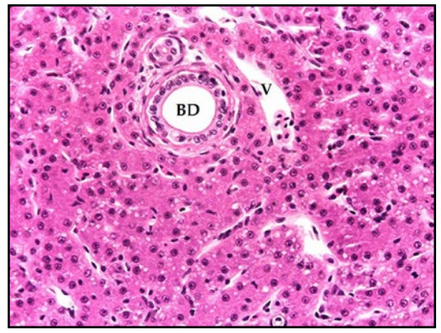


Figure 38. Green grouper (*Epinephelus coioides*) liver (H&E): bile duct (BD) and hepatic vein (V).

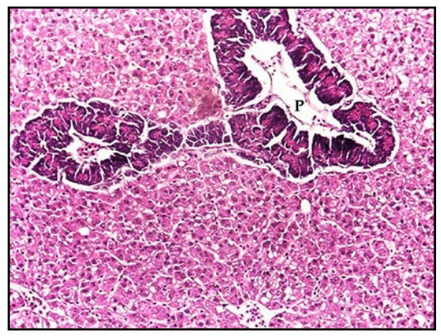


Figure 39. Seabream (*Pagrus* sp.) liver (H&E): pancreatic islet (P).

The nutritional stage of a fish can be gauged by the amount of glycogen in the liver using the Periodic Acid-Schiff (PAS) stain which stains glycogen pink

inside hepatocytes. There are several features of liver pathology to take note of in fish –

- Pleomorphic hepatocyte nuclei, some quite enlarged due to failure of division which can be due to hepatotoxins.
- Eosinophilic deposition indicative of degenerative change similar to that in the kidney tubules

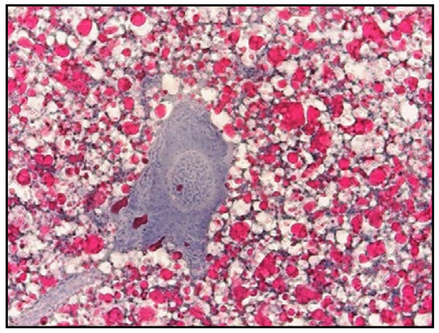


Figure 40. Red drum *(Sciaenops ocellatus)* with fatty liver; Oil Red.

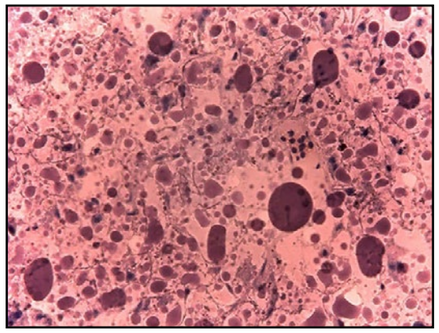


Figure 41. Green grouper *(Epinephelus coioides)* with fatty liver; Sudan Black.

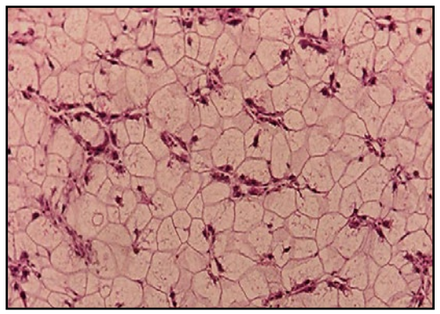


Figure 42. (H&E) Green grouper (*Epinephelus coioides*) with fatty liver degeneration, showing pyknotic nuclei (no distinct nucleolus and condensed).

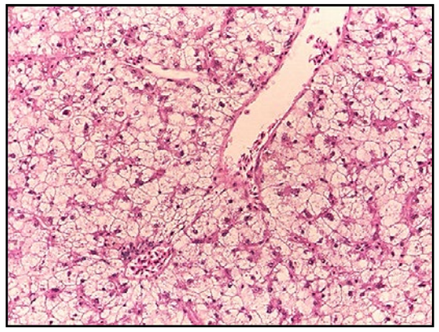


Figure 43. Brown spotted grouper (*Epinephelus areolatus*) with fatty liver degeneration showing disrupted hepatocyte cell membranes and pyknotic nuclei.

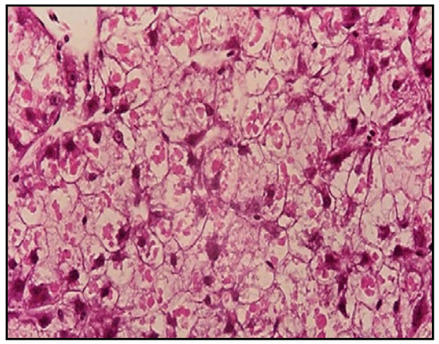


Figure 44. (H&E) Green grouper (*Epinephelus coioides*) with hepatocellular fatty degeneration; note the cytoplasmic eosinophilic droplets and cell membrane disruption.

Spleen

Histology of the fish spleen (Fig. 46) is important in the assessment of the following:

- Immune status – haematopoeitic and lymphopoeitic tissue depletion is a sign of immunosuppression.
- Exposure to disease – presence of pathogens and melanomacrophage centres (Fig. 45).
- Haemal status – presence of haemosiderin is a sign of increased erythrocyte loss.

The spleen is basically a filter organ for the blood circulation, a site for processing of effete erythrocytes. Hence it consists of a reticular network of sinusoids held in place by a capsule which allows expansion of the organ when congested with blood.

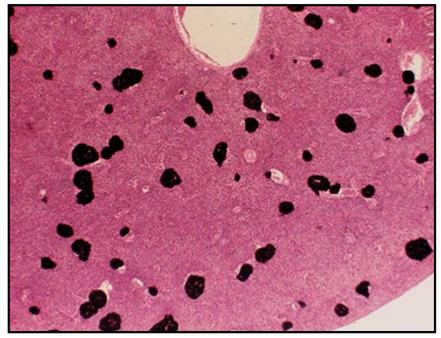


Figure 45. (H&E) Pompano (*Trachinotus blochii*) spleen with MMCs.

Splenic congestion may occur in infections or as a result of stress associated with increased cardiovascular output, but the normal spleen will have a level of blood filling so care is needed not to over interpret what is a physiological response. TIP note: if the spleen is grossly swollen or dark, or histologically the reticular walls of the ellipsoids are obscured and there are areas of haemorrhage or blood pooling, then this is likely to be pathological splenic congestion (Fig. 47).

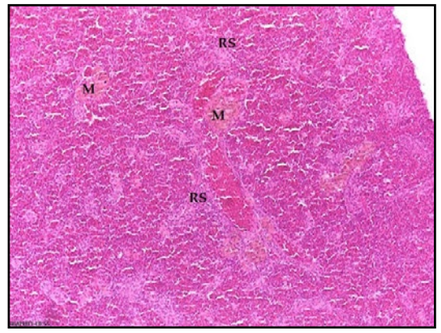


Figure 46. (H&E) Goldfish (*Carassius aurata*) normal spleen with fairly distinct reticular sinusoids (RS) containing blood and a few light brown normal looking MMCs (M).

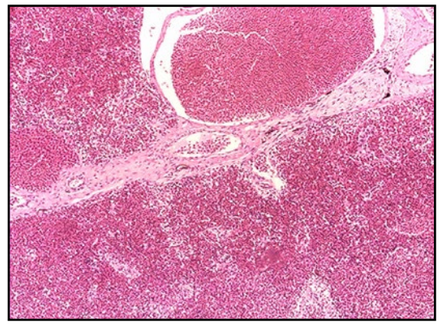


Figure 47. (H&E) Green grouper (*Epinephelus coiodes*) with splenic congestion; note blood pooling in the vein.

Necrosis of the spleen may indicate viral infections particularly of the haematopoeitic cells. Granuloma formation can mean chronic parasitic or bacterial disease (Fig. 48).

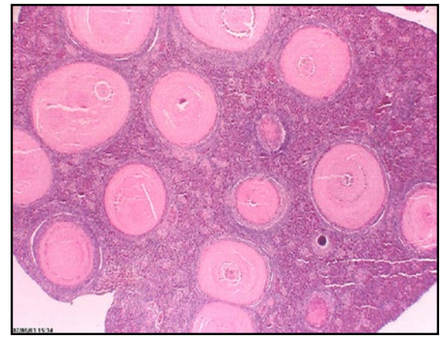


Figure 48. (H&E) Multiple large granulomas in goldfish (*Carrasius auratus*) spleen.

Heart

The fish heart has the following histological features :

- Pericardium – thin connective tissue sac closely adhered to the myocardium, retaining a very small space for some pericardial fluid.
- External myocardium – a layer of relatively compact myocardial muscle fibres.
- Internal myocardium (Fig. 49) – a trabecular structure of myocardial muscle fibres. Together with the external myocardium, it forms the ventricle. The ventricular chamber is then criss-crossed by the trabecular myocardium.

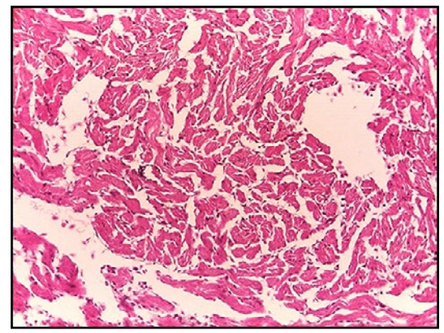


Figure 49. (H&E) Brown spotted grouper (*Epinephelus areolatus*) ventricular internal myocardium.

- Endocardium – lines the myocardial fibres and has macrophages which 'screen' the blood for pathogens and antigens. TIP note: in a bacteraemia, these macrophages can be filled with phagocytosed bacteria. Therefore the heart is a good site for bacterial culture.
- Valves – these are located at the sinoatrial and bulboventricular locations and potentially can be sites of thrombosis.
- Bulboarteriosus – it consists of highly elastic collagen which stains bluish purple in H&E.
- Atrium – it is much thinner than the ventricle, having very thin myocardial fibres, and considerable numbers of endothelium-based macrophages (Fig. 50). Tends to hold more blood than the ventricle.

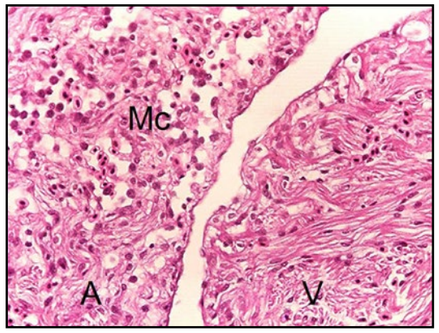


Figure 50. (H&E) Red snapper (*Lutjanus malabaricus)* heart: atrium (A), ventricle (V) and macrophages of the endothelium (Mc).

Gastrointestine

The glandular stomach of carnivorous fish species consists of acid secreting acinar glands in the folds of the gastric mucosa, mucus glands, submucosa connective tissue and muscular layers (Fig. 51). The gastric muscle consists of an inner (granulosa) and an outer compact layer. The gastric glands should be full with clear secretions in an actively feeding fish. Mucus produced by the mucus glands coats the gastric mucosa, protecting it from acid digestion. The gastric glands are mainly in the fundic portion, the mucus glands in the cardiac portion. The pyloric caeca, in species that have them, are finger-like projections of the stomach and have a circular cross-section (Fig. 52). These have very long villi projecting into the lumen. In between the ceca is the visceral fat and interspersed portions of the pancreas. In the lamina propria of the stomach is located fixed eosinophilic granulocytes (mast cell equivalents) which can respond to antigens that may cross the epithelial barrier of the stomach (Fig. 53).

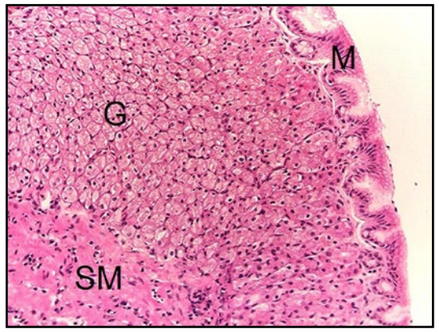


Figure 51. (H&E) Gastric mucosa (M), gastric glands (G) and submucosa - muscle layer (SM) of brown spotted grouper (*Epinephelus areolatus*).

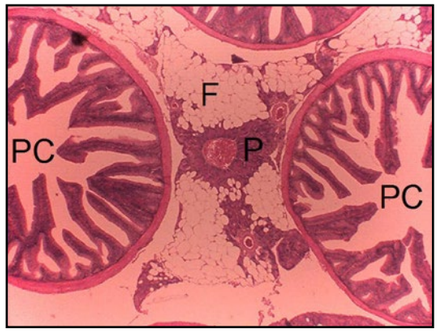


Figure 52. (H&E) Pyloric caeca (PC), visceral fat (F) and pancreas (P) of brown spotted grouper (*Epinephelus areolatus*).

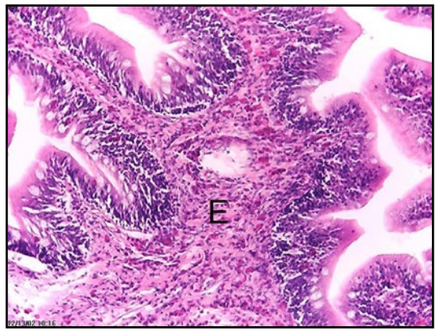


Figure 53. (H&E) Eosinophilic granulocytes (E) in the lamina propria of the pyloric caecal villus of yellow-finned seabream (*Pragus* sp.).

The intestines are tubular in cross-section and consist of an epithelium with cuboidal to columnar epithelium supported by a lamina propria in which are considerable numbers of lymphocytes. TIP note: an enteritis is noted when there is a large increase in the numbers of lymphocytes – these may be focally distributed and migrate into the epithelial cell layer in much greater numbers. Location of the inciting cause, e.g., Parasitic coccidia in large numbers intralesionally, would be diagnostic. However, many gut parasites produce no apparent pathology and are thus incidental. The intestinal lumen can increase in size considerably when food is present. The intestinal villi should be present and sloughing of the epithelium can indicate ingestion of toxins or the presence of infections leading to faecal cast formation and trailing grossly. However care is needed to differentiate this from post-mortem change.

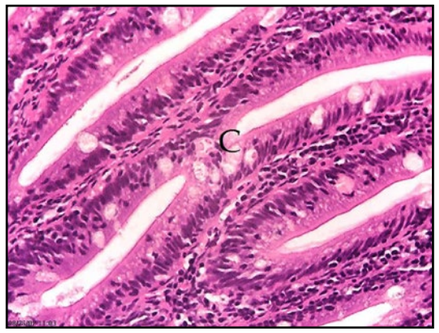


Figure 54. (H&E) Koi carp (*Cyprinus carpio*) intestinal villi; note focal coccidial cysts (C) in the mucosa and the presence of lymphocytes in the submucosa and epithelium.

Parasitic and mycobacterial granulomas may be present as chronic infections, usually in the submucosa. Whole helminth sections may also be found in the intestinal lumen – indicative of adult worms and the fish being the definitive host. Although in *Anisakiidae*, larval stages of the nematode may be intra-luminal when they are ingested within feed organisms (crustacea), the third-stage larvae are released and then penetrate the fish gut wall (Woo and Buchmann, 2012). The intestinal epithelium is constantly renewing itself, and remnants of effete epithelial cells appear as bright eosinophilic inclusions in the mucosa (Fig. 55), especially during periods of starvation. These are apoptotic cells adjacent to viable cells and are phagocytosed by adjacent enterocytes (Ferguson, 2006).

The pancreas consists of exocrine acinar cells and islet of Langerhan endocrine cells (Fig. 57). In healthy, feeding fish there should be zymogen granules in the acinar pancreas (Fig. 58). These are strongly eosinophilic (almost refractile). Severe depletion of zymogen granules may indicate ill-health and inappetance. There is usually fat surrounding the pancreas (Fig. 56). The secretions of the pancreas empty into ducts which connect to the intestinal lumen.

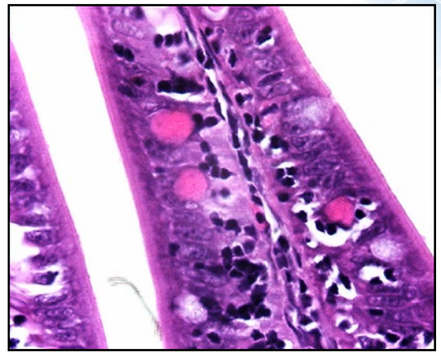


Figure 55. (H&E) Pompano (*Trachinotus blochii*) intestinal villus with effete epithelial cells seen as eosinophilic inclusions.

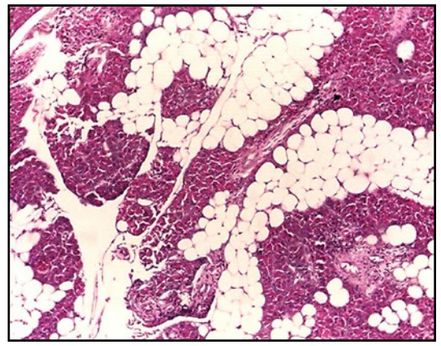


Figure 56. (H&E) Goldfish (*Carassius auratus*) exocrine acinar pancreas with surrounding fat.

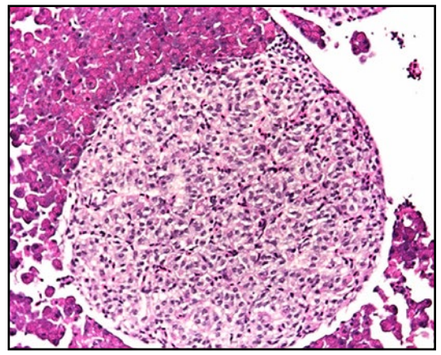


Figure 57. (H&E) Goldfish (*Carassius auratus*) islet of Langerhan.

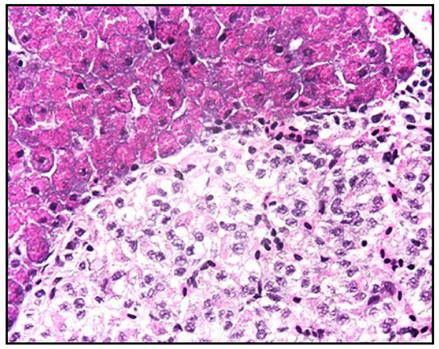


Figure 58. (H&E) Goldfish (*Carassius auratus*) exocrine pancreas with zymogen granules (E) next to islet of Langerhan cells (L).

Brain and Spinal Cord

The central nervous tissue of fish consists of neurons, nerve tracts, their associated sheath tissue and supporting cells for repair and regeneration such as microglia, astrocytes and ependymal cells. Ganglia are groups of neurons that are part of the spinal nerve roots. The key histological feature of the fish brain is its loose, spongy appearance normally compared to the mammalian brain which is more compact in terms of tissue density. Therefore when interpreting vacuolative encephalopathy, one needs to be aware that the brain looks quite 'holey' normally. The distinguishing feature will be the presence of inflammatory cells (lymphocytes, macrophages or astrocytes) and some asymmetry in the lesion distribution. The major portions of the brain includes the optic lobes (quite prominent), the olfactory lobes (cranially located), cerebellum, medulla oblongata (Fig. 59), (the brain stem; which is continuous with the proximal spinal cord) and the hypothalamus-pituitary-pineal portion (Fig. 61). The brain has ventricles containing cerebrospinal fluid and the vasculature consists of small capillaries which in hyperaemia or congestion become much more obvious (Fig. 60).

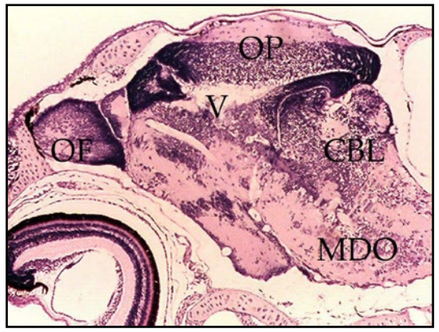


Figure 59. (H&E) Sweetlip (*Lethrinus miniatus*) marine fry brain: olfactory lobe (OF), optic lobe (OP), cerebellum (CBL), medulla oblongata (MDO) and ventricle (V).

A thin meningeal sheath which carries a number of blood vessels covers the brain. Hyperaemia may be a symptom of physiological stress, e.g., as associated with handling or in inflammation associated with infection or if the fish was killed in a way that suddenly increased its blood pressure. The spinal cord has white matter (nerve tracts) and grey matter (neurons). The spinal cord is also very spongy; therefore observation of asymmetry in a transverse section may indicate pathology. The grey and white matter are not as distinct as in mammals (Fig. 62). They contain the nerve roots and centrally located spinal canal carrying cerebral spinal fluid.

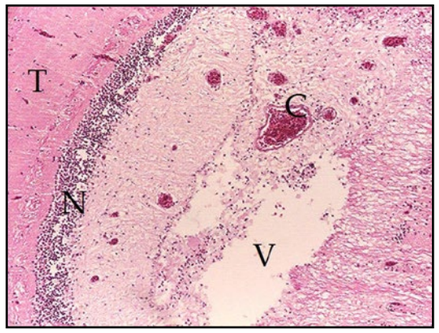


Figure 60. (H&E) Cerebrum of brown spotted grouper (*Epinephelus areolatus*): cerebral nerve tracts (T), neuronal cell layer (N), ventricle (V) and congested capillaries (C).

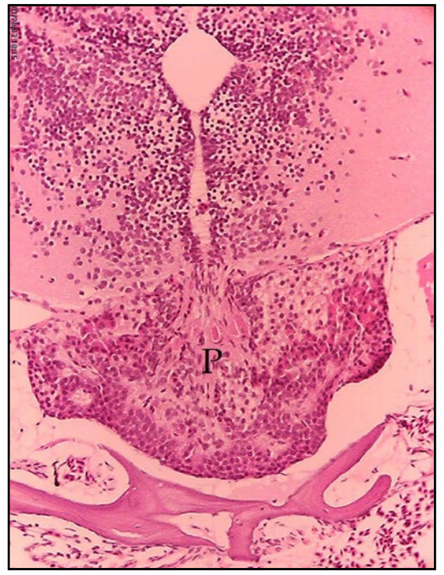


Figure 61. (H&E) Pituitary of Zebra danio (*Dania rerio*) (P).

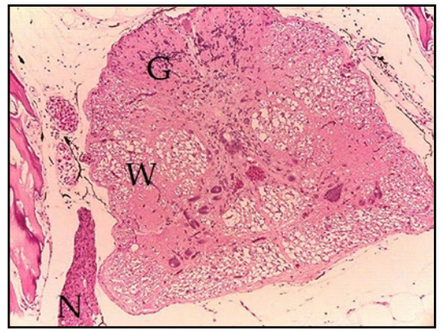


Figure 62. (H&E) Green grouper (*Epinephelus coioides*) spinal cord: grey matter (G), white matter (W) and nerve root (N).

Eye

The fish eye (Fig. 63) is a layered organ consisting of the cornea, conjunctiva/sclera, anterior chamber with aqueous humour, pupil/iris, posterior chamber with vitreous humour, lens (spherical) and the retina. The cornea has five layers – epidermis with external basement, mid-portion (*substantia propria*) and endothelium with internal basement membrane. Cloudiness of the cornea can be due to oedema fluid from ulceration of the epidermis. The pupillary iris tissue is pigmented. The lens is normally laminar (lenticular fibres) and covered by epithelium. In cataracts there is disruption to the fibres (swelling and lysis). The lens is held in place by a suspensory ligament and the lenticular muscle.

The retina (Figs. 64 & 65) consists of the ganglion cell layer (plexiform) inner and outer nuclear layers, the pigmented layer (rods and cones), and the optic disc from which arises the optic nerve. The choroid gland which sits behind the retina is made up of blood vessels and it functions to oxygenate the cornea. The choroid gland can be hyperaemic in disease (Fig. 67). The scleral cartilage provides some rigidity to the eyeball and for histology the eye needs some degree of decalcification (Fig. 67).

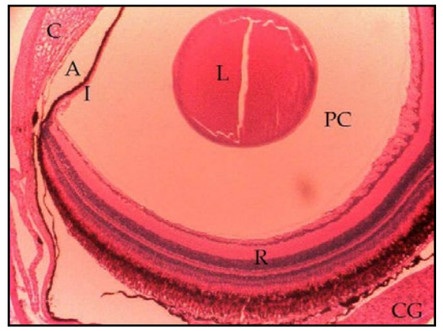


Figure 63. (H&E) Black neon (*Hyphessobryocon herbertaxelrodi*) eye: cornea (C), anterior chamber (A), iris (I), lens (L), posterior chamber (PC), retina (R) and choroid gland (CG).

TIP note: when assessing for vacuolative retinopathy it is important to note artifacts related to sectioning of the eyeball which can create separation of cell bodies in the retinal layers giving a false impression of retinal vacuolation. True retinal vacuolation has vacuoles throughout the layers of the retina with uneven distribution. The vacuole will contain necrotic cell debris (Fig. 66).

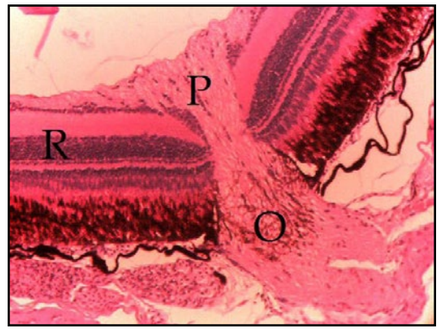


Figure 64. (H&E) Neon tetra (*Paracheirodon innesi*) eye: optic disc (papilla) (P), optic nerve (O) and retina (R).

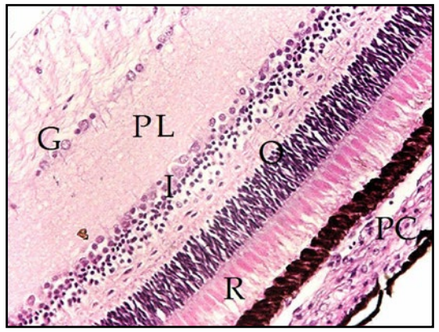


Figure 65. (H&E) Cobia (*Rachycentron canadum*) fish retina: ganglion cell layer (G), plexiform layer (PL), inner nuclear layer (I), outer nuclear layer (O), rods and cones layer (R) and pigmented layer (PC).

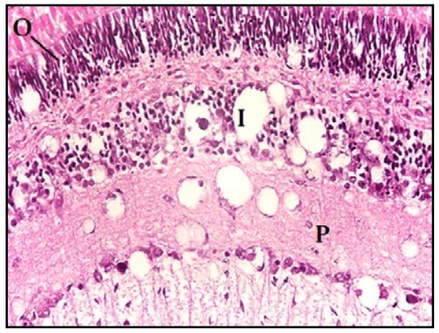


Figure 66. (H&E) Pompano (*Trachinotus blochii*) fish diseased retina; note vacuolation in the plexiform (IP), inner (I) and outer (O) nuclear layers. There is necrotic cell debris in the vacuoles.

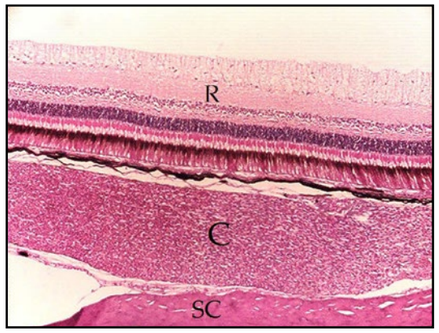


Figure 67. (H&E) Pompano (*Trachinotus blochii*) eye: prominent choroid gland (C), retina (R) and scleral cartilage (SC).

Ovary

The ovarian tissue of fish consists of developing follicles (oocytes), supporting tissue stroma and a thin capsule (Fig. 68). Determining the maturation stage of oocytes relies on the size and vitellogenic content of the oocyte. A mature oocyte which is ready for spawning is large and contains a lot of vitellogenic yolk. After a fish is spawned, there is usually a mopping up of the ovarian tissue involving macrophages, and in this situation debris of atrophied oocytes is seen (Fig. 69).

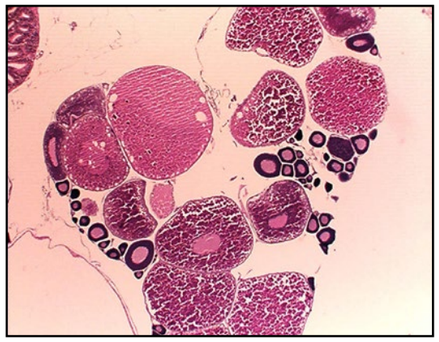


Figure 68. (H&E) Neon tetra (*Paracheirodon innesi*) ovary showing small immature and large mature (ripe) follicles or oocytes held with very thin connective tissue.

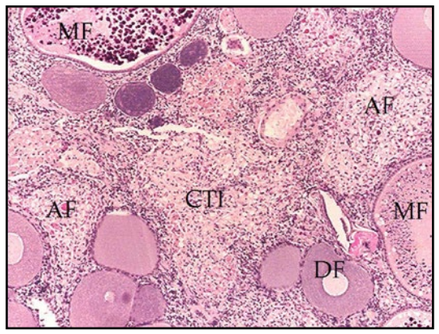


Figure 69. (H&E) Discus (*Symphysodon* sp.) ovary: developing follicle (DF), matured follicle (MF), area of atretic follicular (AF) and contents replaced with connective tissue and inflammatory cells (CTI).

Testes

Fish testes consists of germinal cells that produce the spermatozoa. These are arranged in cords which release the spermatozoa into the vas deferens. Spermatozoa stain intensely basophilic in H&E. They appear as spherical bodies (Fig. 70). The testicular (and ovarian) tissue is subject to disease processes such as parasitism which leads to 'parasitic castration' in severe cases. Spermatozoa travel from the germinal tubules along the vas deferens carried by fluid secretions from the tubular epithelium.

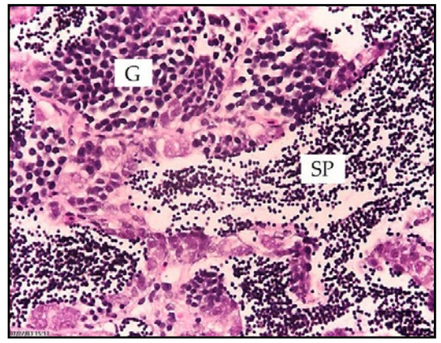


Figure 70. (H&E) Goldfish (*Carassius auratus*) testes: germinal epithelium (G) and spermatozoa (Sp).

Adrenal Tissue

Fish adrenal tissue consists of the interrenal cells located as foci associated with the walls of major blood vessels within the head kidney parenchyma. The interrenal cells are pale, eosinophilic and secrete corticosteroids into the vasculature adjacent to the gland. In chronic physiological stress, the interrenal gland can become hyperplastic or hyperthrophied due to increased demand for corticosteroid secretions (Fig. 71).

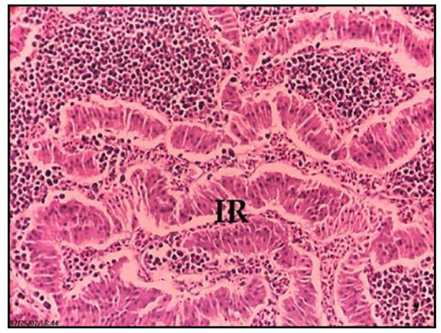


Figure 71. (H&E) Seabream (*Pagrus* sp.) head kidney with interrenal cells (IR).

Thyroid Tissue

Fish thyroid tissue is rather widespread, being located in the branchial area, but also in the head kidney as 'ectopic' thyroid. The thyroid secretion is eosinophilic and fills the glandular space surrounded by the thyroid cell epithelium (Figs. 72 & 73).

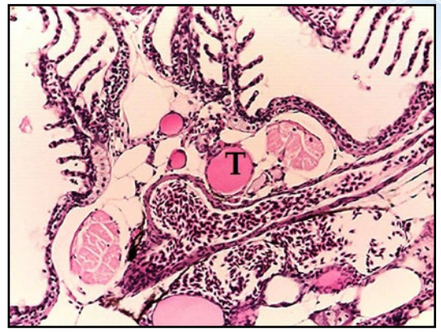


Figure 72. (H&E) Neon tetra (*Paracheirodon innesi*) – thyroid follicles (T) in the gills.

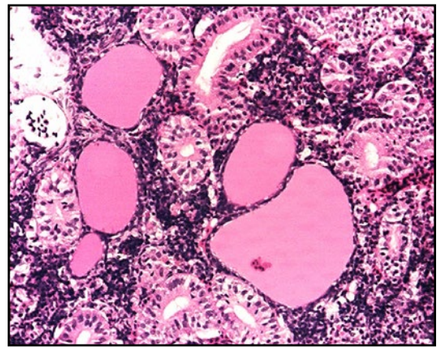


Figure 73. (H&E) Guppy (*Poecilia reticulata*) – renal ectopic throid follicles with proteinaceous thyroid secretions.

Muscle

Fish skeletal muscle and smooth muscle have similar structures to mammals. The skeletal muscle is striated, although not as distinctly as in mammals. Red skeletal muscle has a high content of mitochondria for sustained energy production. White muscle has more 'fast fibres' and fewer mitochondria, suited to rapid anaerobic metabolism. There is proportionately more white muscle than red muscle in fish. Normal skeletal muscle has nuclei located on the periphery of the muscle fibre and there are no 'gaps' or defects in the fibre cross-section (Fig. 74). In myodegeneration, the fibre becomes 'glass-like' in appearance with loss of striated texture and defects occur as muscle protein is degraded as in Vitamin E/selenium deficiency (Figs. 75 & 76). TIP note: artifactual shredding of some muscle fibres can occur if the tissue with skin/scales is not adequately decalcified when sectioned.

Inflammatory processes at the skin level often extend into the underlying muscle via the fascial planes. The inflammation and infection with bacteria and toxin release often cause myonecrosis (Figs. 76a & 76b).

Smooth muscle occurs in the gastrointestinal tract, and in blood vessels (Smith *et al.,* 2006) .They are arranged in layers as circular and longitudinal muscles of the stomach and intestines

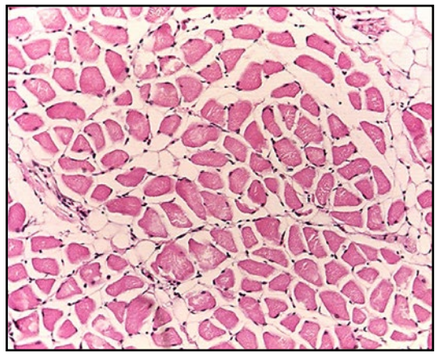


Figure 74. (H&E) Grey mullet (*Mugil cephalus*) – normal skeletal muscle.

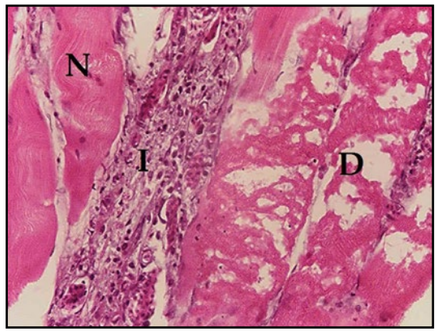


Figure 75. (H&E) Seabream (*Pagrus* sp.) muscle: degenerate fibres on the right (D) and normal fibres (N) left of the inflammatory cell infiltrate (I).

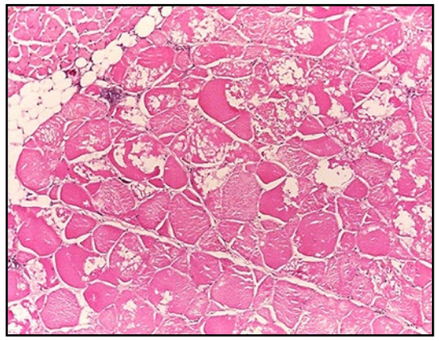


Figure 76a. (H&E) Greater amberjack (*Seriola dumerili*) with coagulative degeneration of skeletal muscle. Take care in interpretation as fixation artifacts can occur (see tip note).

Swim Bladder

This organ consists of a thick collagenous elastic wall with the organ of gas exchange – the rete mirabile or gas gland – located on the inner surface in some species (Fig. 77a).

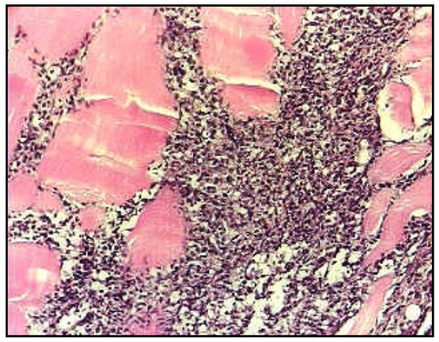


Figure 76b. (H&E) Brown spotted grouper (*Epinephelus areolatus*) – severe myositis; note tracking of macrophages in the muscle fascial planes.

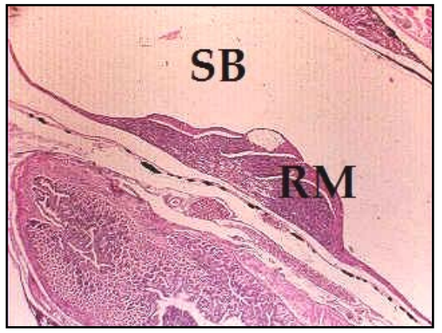


Figure 77a. (H&E) Marine fish swim bladder (SB) with rete mirabile (RM).

Thymus

The thymus consists of densely packed lymphopoeitic cells and is located adjacent to the cranial kidney haematopoeitic tissue. It is well vacularised and is differentiated from kidney tissue by the absence of urinary excretory tubules (Fig. 77b).

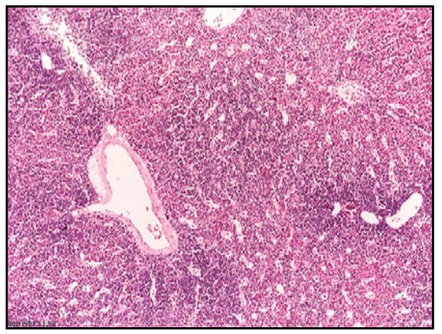
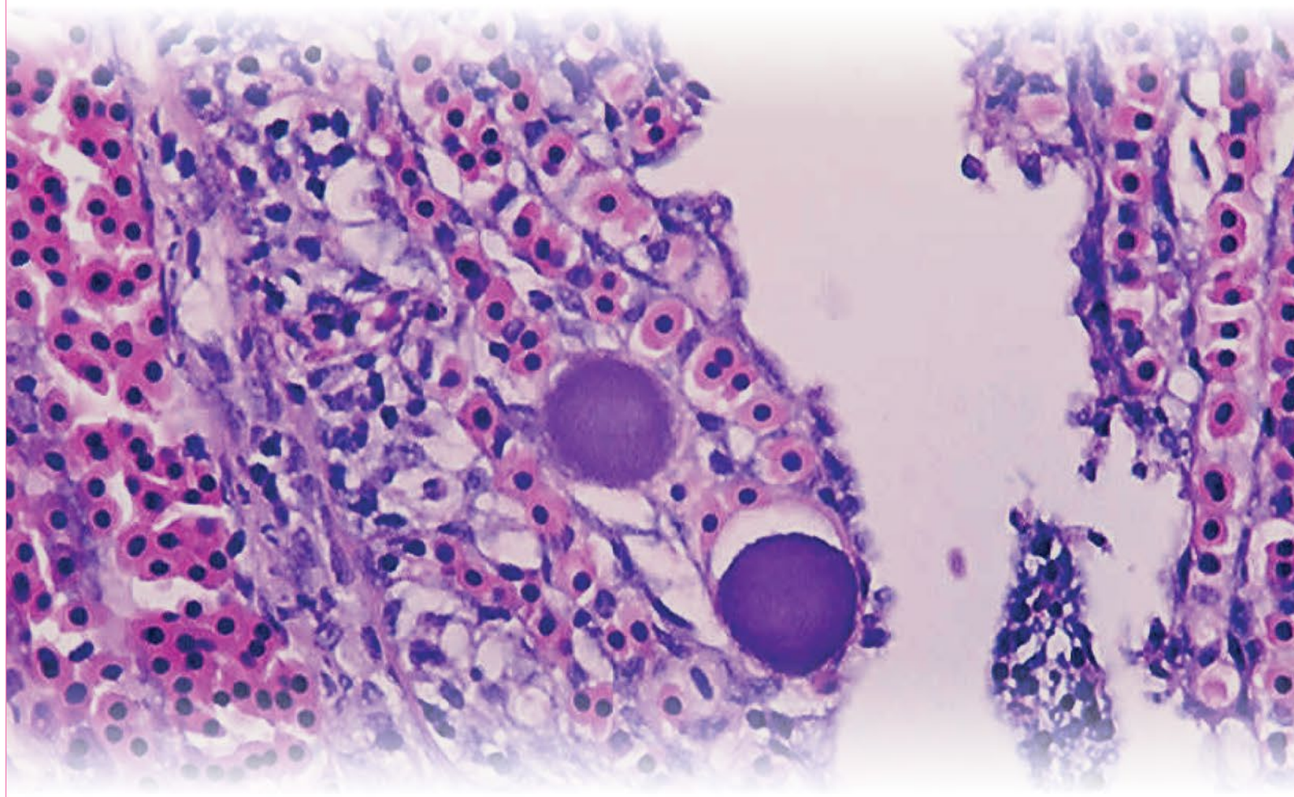


Figure 77b. Russell's snapper (*Lutjanus russelli*) thymus.

PART 2 – DIGNOSTIC APPROACH



PART 2 – DIAGNOSTIC APPROACH

History

Investigations of fish diseases and mortalities begin with the collection of an accurate and specific history from the farmer and/or farm workers as they have first-hand knowledge of the behaviour (feeding, swimming) and husbandry (stocking and movement, grading, harvest, treatments) of the affected fish batches. They would also be able to relay information on recent weather and water quality changes which are important to the development of disease in fish. Typically a form is used to record this information which is submitted with the samples of fish to the laboratory (Fig. 78).

Questions of epidemiological importance in the history include:

- The species and size of fish affected including morbidity and mortality rates.
- The onset and duration of the disease.
- Symptoms of the disease (external lesions and abnormal behaviour).
- Whether the onset of disease is linked to changes in husbandry, water quality or weather conditions.
- Whether the onset of disease is linked to introductions of new fish.
- Whether the disease is spreading from one batch to another (suggestive of an infectious cause).
- The use of any chemotherapeutic agent (which itself can aggravate mortalities or mask important clinical symptoms).

This information will enable the fish health veterinarian or fish pathologist to consider the disease as of either infectious or non-infectious origin. It will also enable the provision of initial health management options aimed at:

- Reducing the impact of active stressors on the affected fish, e.g., reducing stocking density and removal of dead fish.
- Providing biosecurity to other fish stocks through avoiding the sharing of water, feed and equipment.
- Improving water quality through the exchange of clean water and increasing supplementary aeration or withholding of feeding.

These measures are critical for the prognosis of the farmed fish while the results of laboratory testing and pathology are pending.

FISH DISEASE FIELD INVESTIGATION FORM

Name of Farmer	Farm Location		Postal Address		Telephone/Fax Nos.	CASE NO./Date	Staff Signature :
Fish Species Origin Date of Stocking	Number stocked Date of disease	% Affected	% Mortality	Symptoms	Stress Factors	Treatments	Current Status

HISTORY

Figure 78. Fish disease field investigation form.

Sampling

Adequate sampling of fish, water, feed and other environmental samples, e.g., sediment, are crucial to the definitive diagnosis of fish disease and mortalities. Adequate sampling includes:

- A representative number of affected fish.
- Keeping fish alive or proper preservation of fish in fixative.
- Preservation of environment or feed samples in specifically treated containers.

Fish Samples

Poorly preserved samples are the most common cause of failure to arrive at a clinically useful diagnosis for fish diseases, e.g., submission of dead fish (Fig. 82). This is because rapid decomposition of fish occurs post-mortem which renders the sample useless to most diagnostic tests. Live fish samples are most useful diagnostically (Table 3).

Table 3. Examination possibilities with different methods of submission (adapted from Gudkovs (1985))

Method of Submission	Diagnostic Tests
Live	Bacteriology, biochemistry, electron microscopy, haematology, gross pathology, histopathology, parasitology, serology, virology and molecular testing.
Freshly killed on ice (examined within 24 hours)	Bacteriology, virology, molecular testing, limited gross pathology and limited parasitology.
Frozen	Toxicological analysis (preferred method), biochemistry, virology and molecular testing.
Sampled from live, sick fish and promptly preserved samples in appropriate fixatives	Electron microscopy (3% glutaraldehyde fixative), histopathology (10% formalin fixative) and molecular testing (70-90% ethanol).

Fish samples must be kept alive until humane euthanasia prior to post-mortem. They should be kept in their sealed and individually labelled bags that are air or oxygen filled until humane euthanasia. Alternatively battery operated air-line and air-stone aerators are used. They should not be subjected to temperature shocks, i.e., should be kept in insulated styrofoam boxes. In the heat of summer, cooler bricks are included in the boxes to prevent heating of the water as this leads to the fish dying en-route to the laboratory (Figs. 79-81).

Figure 79. Netting and bagging live fish sample.

Figure 80. Oxygenation of fish sample.

Figure 81. Battery operated aerators to keep fish samples alive en-route to the laboratory.

Figure 82. Dead floating fish are useless as diagnostic samples.

Necropsy Examination

The necropsy examination of fish is a discipline necessary for the assessment of gross pathological changes, collection of tissue subsamples for microbiology, histopathology or toxicology and parasitology. No necropsy procedure is perfect for every diagnostic situation; however the following routine used by the AFCD fish diseases laboratory covers most sampling contingencies required.

1. Ideally live, moribund fish should be submitted in order to gain the most diagnostic information from the necropsy (Fig. 85). If a dead fish is submitted or when live samples have died on arrival, it must be removed from the water and wrapped in moist paper or plastic wrap to prevent drying. It must be refrigerated or placed on ice while preparations are underway for the necropsy. Do not freeze the specimens except for toxicological analyses. All fish submitted for necropsy must be processed as soon as possible to minimise rapid decomposition of fish tissues following death.

2. Prepare the necropsy work station(s) by ensuring the following disinfecting; dissecting and sampling equipment (Table 4, Fig. 83) are readily accessible. Disinfect the workstation by wiping down the bench area with 70% ethanol. All staff doing necropsy work must wear sterile gloves, clean facemasks and laboratory coats.

Table 4. Finfish necropsy equipment

Anaesthetic agent – MS 222 powder
Dissection board (polyurethane for ease of disinfection)
Dissecting instruments – scalpel handles and blades, forceps and scissors in a range of sizes from large Mayo to fine iris type, bone cutters and hand saw (for large fish)
Kidney dish to hold instruments with disinfectant
Syringes – 1,3 or 5 cc
Needles – 25 or 27 gauge, 1 inch
1.5 ml microcentrifuge tubes
15 cc plain centrifuge tubes
Blood tubes – plain serum tubes, lithium heparin tubes and fluoro oxalate glucose tubes
Microscope slides and cover slips
Saline or distilled water for wet mounts
Pipettes
Petri dishes
70% alcohol
Sterile stick swabs
Portable alcohol candle flame
Portable light source
Dissecting microscope
Compound microscope
Permanent marking pens and labels
Disinfectant (Betadine – 1% Povidone iodine)
Sterile plastic containers with 10% neutral buffered formalin (saline or distilled diluted) for histology
Digital camera and floppy disks
Paper towelling
Disposable gloves
Face masks
Waste disposal plastic bag-bucket
Weighing scale
Ruler

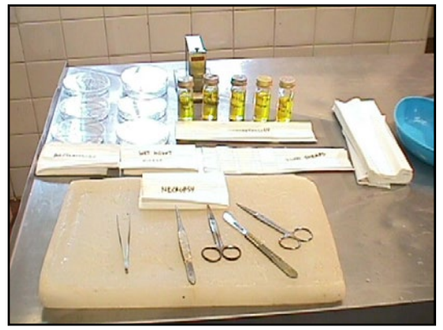


Figure 83. Fish necropsy instruments.

3. Each necropsy must be performed in a systematic and consistent sequence. The protocol in Table 5, which is detailed in the following paragraphs, must be followed for all finfish necropsies. The duty fish pathologist on a case-by-case basis may require additional examinations. This ensures that all organs are

examined and appropriate samples taken. For small ornamental fish, modifications to the necropsy technique are required, and a dissecting microscope should be used.

Table 5. Finfish necropsy examination

External Examination

1. Species Identification
2. Weight and length
3. General condition
4. Blood collection
5. Fins
6. Skin scraping - (This should be done as soon as the fish have been euthanased)
7. Eyes and nares
8. Oral cavity
9. Anus

Internal Examination

1. Gill biopsy wet mount exam and sampling for histology (this should be done as soon as the fish have been euthanased)
2. Remove the eye
3. Remove the brain
4. Disinfect
5. Open the body cavities
6. Remove the heart
7. Remove the abdominal body block
8. Dissect out the liver
9. Dissect out the gall bladder
10. Dissect out the spleen
11. Remove the gonads
12. Remove the swim bladder
13. Remove the cranial and caudal wedges of the kidney, vertebrae, spinal cord and muscle
14. Sample the skin, including the lateral line and nares
15. Open and sample the stomach and intestines

4. Note down the common or species name of the fish species.

5. *Weight and length* – Record the weight in grams/kilograms. There are different measures of length. The standard length is snout to last vertebra in the tail; fork length: snout to "v" of fork in the tail fin, or centre line of the fin if there is no fork; total length = snout to posterior-most tip of the longest tail fin (as with eels for example). Record the total length unless there is tail-fin erosion, in which case the fork length may be measured. Indicating which measurement is taken minimises errors in interpretation.

6. *General condition* - Examine all the external surfaces, noting loss of scales, ulcers, areas of discolouration, masses or other abnormalities. The location of all lesions is important and must be noted. (A lesion is defined as any area or part of any organ or tissue showing pre-damage in some ways.) Look at the body form and palpate to determine if there is any muscle wasting, ascites (fluid causing swelling of the abdomen) or skeletal deformity. Note any spinal curvature – scoliosis (lateral or sideways curvature) or lordosis (dorsoventral or hunch-back curvature). Also note the age of the fish – fry, fingerling, juvenile or adult.

7. *Blood sampling* - (Fig. 84) With the fish still alive but in deep sedation, blood is drawn from the tail vein by ventral insertion of a sterile 25-27G gaugeneedle and 1-3 ml syringe to collect as much blood as available (usually 0.5 ml in small fish, 1-2 ml in large fish). Alternatively, blood may be drawn by severing the gill arch (es) and collecting by capillary tubes. (After blood collection, the fish is euthanased by severing the spine behind the head.) Empty the blood from the syringe after removing the needle from the capillary tubes by gravity into sterile blood tubes as follows: 1. Plain tube for serum enzyme and electrolytes (Na^+, K^+, Cl^-) analysis. 2. Lithium heparin tube for haematology. 3. Fluoro-oxalate tube for glucose analysis. Gently rotate heparin and fluoro-oxalate tubes to ensure proper anticoagulation of the samples. The serum sample is obtained by allowing the blood to clot and then centrifuging to separate serum from blood cells. Serum is siphoned into clean Eppendorf vials. Blood and serum samples are labelled and delivered to Path Lab Medical Laboratories for analysis. The Case Manager will select the tests required.

Figure 84. Tail vein blood sampling.

8. *Fins* – Examine all of the fins for fraying, erosion, necrosis or small-discoloured spots that may be parasites. Check the fin bases for reddening or haemorrhage. Look for nodules, lumps and growths on the fins, which may suggest *lymphocystis* disease. Check for *Saprolegnia* fungal infection, which looks like white cotton wool-like growths. Scrape such lesions for wet mount examination. Sample such lesions for histology.

9. *Skin scraping* – Prepare a skin scraping with a clean scalpel, evaluating both normal skin and the edges of any lesions. (Do this as soon as possible after euthanasia of the fish – otherwise most parasites will leave the skin.) The scraping should be deep enough to remove a few scales in addition to the mucus layer. Do the scraping early on in the necropsy as parasites leave the dead fish quickly and surface bacteria proliferate. Place the scraping on a clean microscope slide with a drop of water and under a clean cover slip for examination. Examine the wet mount for parasites. Do a parasite count estimate. Check also for bacteria, especially motile or filamentous bacteria like (*Flexibacter* spp.*).* Do a smear of skin lesions for Gram staining.

10. *Eyes and nares* - Examine the eyes for corneal opacity or exophthalmia. Also examine the anterior chamber for the presence of blood or exudates. Note the lenses – opacity indicates cataracts. Check for parasites such as eye flukes inside the eyeball. The nares (nostrils) can be examined by pressing the area behind them. Culture any discharges for bacteria. Remove the nostrils and head sinus block for histology.

11. *Oral cavity* – Open the mouth and look inside. Note any teeth, lumps or discoloured areas. Check for parasites – worms. Look for inflamed tissue – which is red and swollen.

12. *Anus* - Examine the anus for swelling, redness or ulcerations. Look for any protruding worms or faecal trails. Do a wet mount of faecal trails looking for protozoan parasites.

13. *Gill biopsy wet mount exam and sampling for histology* – This must be done as soon as possible after the fish is euthanased to avoid the loss of parasites from the gills and the rapid onset of autolysis. Gill pathology is critical in the diagnosis of 30% of fish cases. Cut away the operculum (gill cover) to expose the gills. The pseudobranch is a bright red gill-like organ on the underside of the gill cover and should be removed for histology.

    Remove the second and third gill arches for histology as these may better trap parasites than the 1st gill arch. The first gill arch is uppermost when you open the gill cover. The gills should be bright red in fresh specimens. Fish submitted for necropsy longer than 1 hour after death often have very pale or white gills and are unsuitable for histology owing to autolysis. Fresh fish with pale gills may be anaemic, have serious fluid imbalances or the blood has drained away from the gills. Look for any abrasions, excess mucus or parasites (e.g., white spots) and foreign material (e.g., sediments, algae).

    Snip off about 10 – 20 primary gill filaments close to the gill cartilage or a whole gill arch in very small fish. Do not include the gill cartilage unless it is a very small fish for proper wet mount examination. The cartilage tends to interfere with even placement of the cover slip so that the filaments do not lie flat for proper viewing. Under a light microscope, check for gill filament hyperplasia (thickening with more cells) or fusion of filaments, filament necrosis, bacterial/fungal infection, gas emboli or blood aneurysms. Gauge the amount of mucus and identify motile or attached protozoan ciliates, flagellates and monogenean flukes. Slight pressure on the cover slip may loosen gill parasites for better examination. Also note any foreign material in the gills. Do Gram, Ziehl-Neelsen and Giemsa stains on smears of the gill filaments.

14. *Remove the eye* – With forceps, grasp the eye by the conjunctiva and gently free it from the surrounding skin with a pair of fine scissors. Pull gently on the globe and cut the underlying muscles. Examine the orbit for exudates (pus

or fluid), haemorrhage or masses. At least one eye should be fixed for histology. The other eye can be opened to evaluate the lens more closely. Parasites such as the trematode *Diplostomum spathaceum,* may be located in the lens and cause herniation of the lens. If pop eye occurs, use a sterile needle-syringe to draw out fluid from the eyeball for bacterial culture or submit the whole eye.

15. *Remove the brain* - The brain can be examined by dissecting away the roof of the skull. First cut behind the skull and with scissors or a saw, cut on either side of the skull to lift the roof off the brain. The brain is normally white and firm. Note any haemorrhage or discolouration of the cerebrospinal fluid or the brain itself. Then cut the spinal cord and tease the brain off the floor of the skull by using the blades of a pair of scissors. Brain autolyses quickly so remove it early in the necropsy for fixation. Split the brain longitudinally in half – use one half for virology or bacteriology, the other half for histology where applicable if only one fish is available.

16. *Disinfect* – Before dissecting the abdomen disinfect the surface of the fish with 70% alcohol. Place the fish in the right lateral recumbency on the dissecting tray – use a non-slip surface, e.g., moist paper towel or disposable sponge if necessary.

17. *Opening the body cavities* – Using a sterile technique, make an incision starting at the operculum. Cut through the pectoral girdle (bony part under the pectoral fins) parallel to the ventral midline. Extend the incision caudally and cut through the pelvic girdle, next to the anus and then dorsally to and along the spine parallel to the lateral line. Meet up with the incision at the operculum. Remove the body wall. Watch for adhesions from any organs to the body wall.

    Once the contents of the pericardial cavity and the abdomen are exposed, culture any fluid in the body cavity and record its volume, colour and consistency (thick or clear). Note the size, colour and consistency (firm or soft) of each organ in-situ and how much fat tissue is lying between the organs.

    Before disturbing any organ, culture any tissue that appears abnormal. If the abdomen has been contaminated during entry, flush the target organ with 70% alcohol. A sterile scalpel blade will be used to make an incision in the infected organ. A sterile disposable plastic loop or a metal flamed loop (until red hot) is then inserted into the cut organ to collect tissue sample, which is inoculated onto bacterial culture media.

    Organs or parts of organs for bacterial culture may also be entirely removed by preflamed forceps and scissors and kept in sterile Eppendorf vials for the fish bacteriologist to inoculate onto culture media. Inoculation must be done on the same day as the sampling.

    Then remove the organs for closer examination and collection of histological specimens, impression smears or squash preparations. With very small fish for histology (less than 3 cm in length), remove one gill cover and one side of the abdominal wall as well as opening the skull to allow fixative to penetrate before placing in the fixative. Ensure that tissue segments for histology do not exceed 1 cm x 1 cm x 1 cm in size for effective fixation. Do not put too many tissue parts into one fixative bottle by ensuring that the fixative: tissue volume ratio is at least 10:1. For marine fish, use saline diluted formalin while distilled water formalin should be used for freshwater fish.

18. *Removing the heart* – The heart is found between and just behind the gills. The elastic, whitish bulbus arteriosus is attached to the ventricle. Grasp the bulbus arteriosus, pull caudally and cut the connection to the ventral aorta. Then lift the heart out of its cavity and cut the dark red coloured sinus venosus.

    Examine the surface of the heart for any raised or discoloured lesions. A large heart can be cut open to examine the endocardial surface and valves. Look for any adhesions and nodules on the valves or walls.

19. *Removing the abdominal body block* – This is to remove all the stomach, liver, spleen and intestines in one block. First cut the oesophagus. Grasp it and pull out these organs. Free them by cutting the intestine at the anus. The swim bladder and gonads may also be removed by this action. Place the block on a clean petri dish for further dissection.

20. *Dissect out the liver* – Free the liver from the surrounding tissue. Examine the edges, which should be sharp. Note the colour. The liver is usually reddish tan, but can show marked variations in colour, becoming yellow pale in fish raised on some fatty diets. Make several cuts into the liver and examine the colour and texture of the cut surface. Parasites such as cestode plerocercoids (tapeworm larvae) appear as small white lines or spots. If you see lesions, take a section to examine as a wet mount and save the rest for histology. Take care to hold the liver gently by the edge rather than gripping in the main lobes, as this will fragment the organ as it is fragile.

21. *Dissect out the gall bladder* – The gall bladder is a 'green' sac located between the liver lobes and may be full if the fish has not been eating. Note the colour of the bile juice, which may be dark green to straw yellow. Bile can be sampled by a syringe for toxicological analyses.

22. *Dissect out the spleen* - The spleen is usually a flat, triangular, dark red organ located amongst abdominal fat near to the stomach. If the spleen is swollen and round it should be cultured aseptically. Then cut the spleen and examine the cut edges; place a section in fixative for histology.

23. *Removing the gonads* – The gonads (testes or ovaries, eggs) are usually located in the caudal abdomen and may not be grossly visible in immature or sexually inactive fish. If the gonads are visible, note their size, colour and consistency. Make several incisions into the gonads and evaluate the cut surface. Note the state of development of the eggs (inactive, developing or mature). Mature ova are usually bright yellow or orange while mature testes are large and milky white. Immature but developing ova are often a greenish colour. In fresh samples, sperm may be examined for viability and motility.

24. *Removing the swim bladder* – The swim bladder if present is a white or clear hollow organ located dorsally along the spinal column. It is usually identified if it has not been ruptured. The number of chambers varies as can the prominence of the red gas-forming organ. If the swim bladder is present, gently open it watching for any exudates or haemorrhagic areas. Be ready to take cultures if this is the case. The swim bladder can fail to develop or inflate in some hatchery-reared fish. This may be associated with skeletal deformities. Over-inflation also occurs. Include part of the swim bladder in samples for histology.

25. *Removing a cranial and caudal wedge of kidney, vertebrae, spinal cord and muscle* – Examine the kidneys next. The kidneys of fish are retroperitoneal (sit outside the peritoneal cavity, covered by a sheath of fibrous connective tissue), lying next to the spinal column, dorsal to the swim bladder. They are dark reddish brown and may be distinctly lobed or intimately embedded along the vertebrae. The cranial (head) kidney may be separate or fused with the caudal (tail) kidney. A urinary bladder may be present near the anus.

26. If septicaemia is suspected, culture the kidney (usually head kidney) because bacteria tend to localise in it. First flush the kidney sheath with 70% alcohol, then cut the sheath with a sterile scalpel and follow with a flamed loop to collect kidney tissue for bacterial culture. Perform a smear of the kidney, (cut away the fibrous sheath first) for Gram, Ziehl-Neelsen and Giemsa stains to examine for bacteria, mycobacteria and cytology.

27. Kidney samples for histology should include parts from the head and tail kidney. For fish with large lobed kidneys, e.g., goldfish, sample a portion. For fish with small or tightly attached kidneys (to the spine) cut a wedge(s) section of tissue to contain kidney, spine and muscle along the dorsal part of the fish. Ensure that the wedge is not larger than 1 cm^3 each for proper fixation.

28. *Sampling the skin, including the lateral line and nares* – Take samples of skin for histology. The sections must include normal skin at the margins of lesions. Cut a strip of skin containing a length of the lateral line and some muscle with it for histology. Cut a section containing the nares (nostrils). After sampling the skin-muscle strip, make several cuts through the body muscle looking for discolouration, softening, white cysts or black spots due to metacercariae and worm larvae.

29. *Opening and sampling the stomach and intestines* - Examine the stomach and intestinal tract. Variations in length and shape of the intestines occur in different species. Some fish have multiple pyloric caeca (e.g., grouper and snappers), others may have a spiral intestine (e.g., goldfish) and others may have a short and straight gut. Note any

raised or haemorrhagic areas on the serosal surfaces. Cut open the lumen and examine the contents of the entire tract. If undigested material is in the stomach, try to identify what the fish was eating. Note any irregularities in the mucosal surface. Scrape the stomach and intestine contents onto a microscope slide for wet mount examination. For very small fish, include a section of the gut for wet mount examination. Finally, take sections of the stomach, pyloric caeca (if any), and small and large intestines for histology. For very small fish, take the whole gastrointestinal tract for histology.

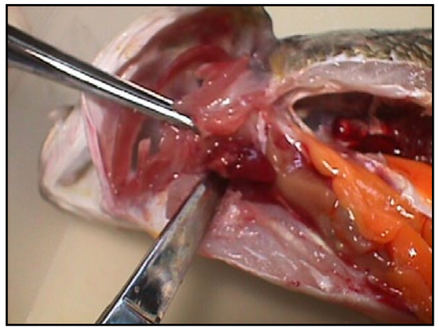


Figure 87. Sterile and sharp dissection instruments important for fish dissections.

Figure 85. Performing fish necropsies requires a team effort to ensure that specimens are sampled as fresh as possible.

Figure 86. Aseptic techniques in fish necropsy.

Bacteriology

Bacterial culture and identification of fish diseases is based on the same principles as mammalian microbiology. These are:

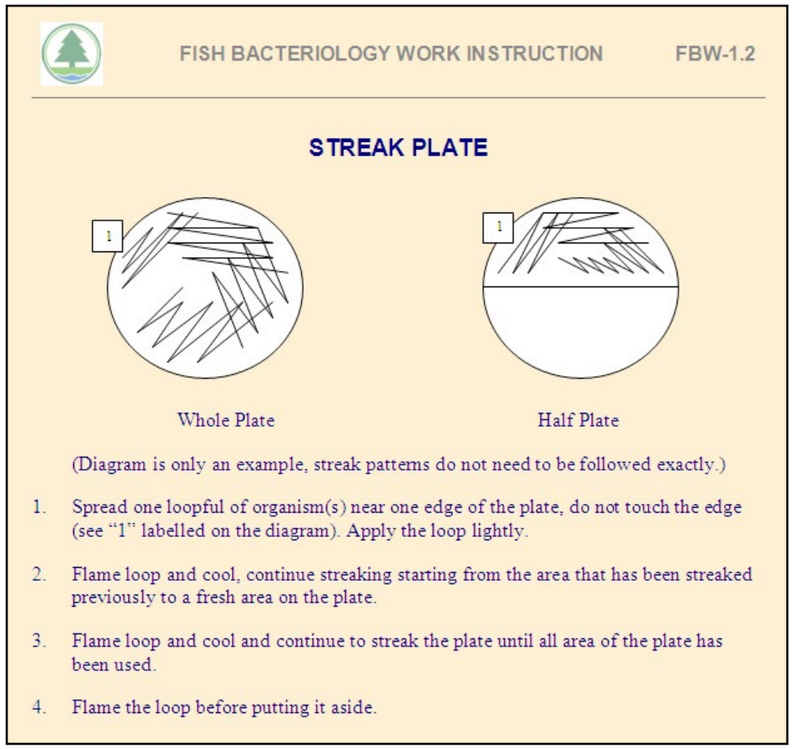
- Aseptic collection (Fig. 86) and plating of tissue samples.
- Use of basic culture media, e.g., blood agar, trypticase soy agar for primary culture.
- Use of selective media which enhances the culture of particular groups of bacteria which have unique nutrient requirements for growth and isolation.
- Incubation conditions include temperature, aerobic or anaerobic culture to enhance growth of bacterial isolates.
- Characterisation of colony and cell morphology and staining characteristics (e.g., Gram, acid-fast)
- Biochemical activity profiling for speciation of isolates.
- Antibiotic sensitivity testing (antibiogram).
- Immunological tests.

Sample Collection

Asepsis begins during necropsy to avoid contamination of the kidney, spleen, heart or lesion samples with external skin or internal gut flora of bacteria. Flaming of instruments and alcohol surface decontamination before taking of tissue to be inserted into sterile vials is required (Fig. 87). Contamination of samples usually leads to growth of a very mixed colony of bacteria on the plate, making it almost impossible to select the dominant colony(ies) for further identification. This is a particular problem in fish due to the presence of water (and normal flora bacteria) over the entire external surface of the fish. Once sampled, the sample vial must be accurately labelled to enable the results to be valid.

Plating

Samples from diseased fish are individually plated, i.e. one tissue type per plate of media (Fig. 88). The media used need to be freshly prepared and sterilised before use.



FISH BACTERIOLOGY WORK INSTRUCTION FBW-1.2

STREAK PLATE

Whole Plate Half Plate

(Diagram is only an example, streak patterns do not need to be followed exactly.)

1. Spread one loopful of organism(s) near one edge of the plate, do not touch the edge (see "1" labelled on the diagram). Apply the loop lightly.
2. Flame loop and cool, continue streaking starting from the area that has been streaked previously to a fresh area on the plate.
3. Flame loop and cool and continue to streak the plate until all area of the plate has been used.
4. Flame the loop before putting it aside.

Figure 88. Plating work instruction.

Selective Media

Certain bacterial groups require specific nutrient profiles in the media (Fig. 89) to stimulate differential growth out of a sample which may contain a mixture of bacteria. For example, TCBS (thiosulphate citrate bile salt sucrose) is selective for most *Vibrio* spp. Sucrose fermenting *Vibrio* spp. produce yellow colonies while non-sucrose fermenting *Vibrio* spp. produce green colonies (Inglis *et al.,* 1993). Other selective media used is Lowenstein-Jensen used for isolating *Mycobacterium* spp. and KDM 2 media containing L-cysteine for isolation of *Renibacterium salmoninarum.*

Figure 89. Bacterial culture plates – green TCBS, red blood (sheep or horse) agar and trypticase soy agar (TSA) plates (pale beige).

Incubation

Incubation of culture plates under appropriate temperatures is needed for bacterial isolates to grow. For most fish bacteria, the incubation temperature ranges from 15-30ºC, which is quite different from the 37ºC used for mammalian bacteria. To achieve lower incubation temperatures than ambient temperatures, air-conditioned rooms and low temperature incubators are used. Most of the bacterial isolates are incubated in aerobic conditions while specific groups, e.g., *Clostridium botulinum* require anaerobic incubation. This is achieved by using CO_2 gas displacement containers that remove atmospheric oxygen from the incubating container.

Staining

Part of the identification of bacteria requires the use of stains – Gram stain and Ziehl- Neelsen. The work instructions for performing these stains are shown in tables 6 and 7. Gram staining can be done on pure culture isolates, or on smears from diseased tissue samples.

Table 6. Gram Staining

The Gram stain is used to categorise bacteria as Gram-positive or Gram-negative and to observe the cellular morphology. Bacteria will differentially retain the primary stain, crystal violet, based on the characteristics of the cell wall. Gram-positive bacteria, which possess a high content of peptidoglycan and teichoic acid in the cell wall, retain the primary stain and appear purple. Gram-negative bacteria that contain a high content of lipopolysaccharides in the cell wall lose the primary stain during decolourisation. Gram-negative bacteria take up the counter stain, carbol fuchsin, and appear pink.

HEAT FIXATION
Before performing the Gram stain, the smear must first be heat-fixed.

1. Pipet a drop or place one loopful of sterile distilled water or saline on a clean slide.
2. Transfer a small amount of the bacterial colony into the drop of water using an inoculating loop.
3. Mix and spread the colonies with the loop in a circular movement so as to obtain a thin and evenly distributed smear.
4. Allow the smear to air dry and then heat-fix by passing the slide quickly through the low flame of a Bunsen burner 2 to 3 times. (Take care that the slide becomes no warmer than the back of the hand can stand.)

GRAM STAIN PROCEDURE
1. Overlay the smear with crystal violet for 1 minute.
2. Rinse with tap water.
3. Flood the smear with Gram's iodine for 1 minute.
4. Rinse with tap water.
5. Decolourise the smear with acetone alcohol by running the solvent quickly over the smear until no more violet colour is released.
6. Rinse with tap water.
7. Counterstain the smear with carbol fuchsin or safranin for 1 minute.
8. Rinse with tap water.
9. Blot dry with a paper towel or filter paper.
10. Examine the smear under an oil immersion objective for characteristic Gram stain reaction and cellular morphology.

INTERPRETATION
Gram-positive bacteria stain purple. Gram-negative bacteria stain pink.

Table 7. Ziehl-Neelsen Staining

Mycobacterium spp. and *Nocardia* spp. commonly known as acid-fast bacilli are detected in specimens through the use of carbol fuchsin staining. Due to the mycolic acid in the cell wall, *Mycobacteria* are difficult to decolourise after staining. Heat, solvents or detergents are needed to drive the stain into the cell wall of *Mycobacteria*.

Before performing the Ziehl-Neelsen stain, the smear must first be heat-fixed.

HEAT FIXATION
1. Pipet a drop or place one loopful of sterile distilled water or saline onto a clean slide.
2. Transfer a small amount of the bacterial colony into the drop of water using an inoculating loop.
3. Mix and spread the colonies with the loop in a circular movement so as to obtain a thin and evenly distributed smear.
4. Allow the smear to air dry and then heat-fix by passing the slide quickly through the low flame of a Bunsen burner 2 to 3 times. (Take care that the slide becomes no warmer than the back of the hand can stand.)

ZIEHL-NEELSEN STAIN PROCEDURE
1. Flood the heat-fixed smear with carbol fuchsin and heat gently until steaming for 5 minutes. (Replenish stain when necessary and avoid boiling.)
2. Allow the slide to cool and then rinse with tap water.
3. Decolourise the slide with acid-alcohol until no more colour drains from the smear.
4. Stop the decolourisation process by rinsing the smear with water.
5. Counterstain with methylene blue for 2 minutes.
6. Rinse with water and allow to dry.
7. Examine the smear using the oil-immersion objective (100x).

INTERPRETATION
Acid-fast bacilli stain pink, while non-acid-fast organisms and the background stain blue.

Biochemical Profiling

While colony morphology and staining characteristics enable the grouping of isolated bacteria broadly into Gram negative/positive or acid-fast bacteria, biochemical profiling is the next step in the identification of the species of bacteria. Identification is important because the pathogenesis, prognosis and management of a bacterial infection varies according to the phenotype of the bacterial isolate. Bacteria ferment substrates such as sugars and amino acids, and their biochemical reactions are an expression of phenotypic characteristics in certain conditions. A database of these reaction responses and the corresponding bacterial species enables a degree of confidence in naming the bacterium. The API system of biochemical profiling provides a tool for probability-based identification of bacteria using regularly updated databases on the biochemical responses. The API strips containing individualised compartments for each test are inoculated with the isolate and incubated at the appropriate temperature, after which colour change indicates either a positive or negative reaction. The results are then written on the test result sheet and the numerical code corresponding to the set of results is entered into the database for an identification probability list of bacterial species. A species with a high probability (>99% match) is considered as the identified bacterium. While useful, the API database may not contain the fish bacterium of interest, and so, other tables of biochemical characteristics from reference fish bacterial disease texts need to be consulted.

While the API system is convenient to use, it may also not contain all of the biochemical tests of interest, and so, some tests need to be manually set up and run. Examples of such are the Oxidative Fermentation (OF) test (Fig. 90) and the Catalase test (Fig. 91). The important issue to remember is that no single biochemical test is specific to a bacterium species, hence the need to routinely run a series of tests. For this reason, it can take several days before a useful result can be obtained in the isolation and identification of the bacterial pathogen. In the meantime, some control measures must be in place to control the disease at the farm while bacterial culture results are pending; otherwise, for the farmer these results are too late to be useful for his immediate disease management options.

FISH BACTERIOLOGY WORK INSTRUCTION | FBW-3.4

OXIDATION FERMENTATION TEST

Oxidative-fermentative (OF) medium is used to determine if an organism can utilize carbohydrates in an oxidative or fermentative manner. In addition, those bacteria that are nonsaccharolytic, that is, unable to use carbohydrates, can also be identified. As OF medium contains a high concentration of carbohydrate (1%) and a small concentration of peptone (0.2%) which facilitates the oxidative use of carbohydrates by nonfermenting gram-negative bacilli. OF medium can be used as a single test for glucose or as a set of OF carbohydrates such as lactose, maltose, sucrose, and xylose etc. This test is known as the OF test.

Procedure

1. Using an inoculating needle, inoculate 2 OF tubes (for each carbohydrate tested) by stabbing 18- to 24-hour pure colony/colonies into the OF medium.
2. Overlay one of the tubes with 2 ml of sterile mineral oil.
3. Incubate all OF tubes at 22-25°C for 18 to 24 hours. Interpret reactions at 24 hours. If no change has occurred, reincubate for an additional 18 to 24 hours.

Interpretation

Interpretations	Open tube (No mineral oil)	Closed tube (Overlaid with mineral oil)
Nonsaccharolytic	Green or blue-green	Green or blue-green
Oxidative/Aerobe	Yellow	Green or blue-green
Anaerobe	Green	Yellow
Fermentative/Facultative anaerobe	Yellow	Yellow

Figure 90. Work instruction for oxidative fermentation test.

FISH BACTERIOLOGY WORK INSTRUCTION | FBW-3.2

CATALASE TEST

Most aerobes and facultative anaerobes produce hydrogen peroxide when utilizing oxygen. As hydrogen peroxide is toxic to aerobes and facultative anaerobes, some of them possess an enzyme called catalase which converts hydrogen peroxide to water and oxygen. The presence or absence of catalase is one of the important means for differentiation between certain groups of bacteria. For example, the catalase test can be used in differentiating *Streptococcus* (catalase-negative) and *Staphylococcus* (catalase-positive).

Procedure

1. Using a sterile inoculating loop, transfer a well-isolated colony that is 18 to 24 hours old onto a clean microscope slide.
2. Place one to two drops of 3% hydrogen peroxide on the colony. Hydrogen peroxide is stored in the fridge.
3. Observe for the formation of rapid and continuous bubbles.

Interpretation

Catalase-positive: rapid and continuous bubbles formation.

Catalase-negative: lack of bubble formation.

Notes

1. The order in the procedure should not be reversed. Do not add the organism to the reagent. This may lead to a false-positive reaction.
2. Since red blood cells also contain catalase, avoid inclusion of red cells from the medium into the test reaction. This may lead to false-positive reaction.

Figure 91. Catalase test instructions.

Antibiograms

It is essential in fish bacteriology to routinely run antibiotic sensitivity tests on isolates because:

- Bacterial resistance is an issue in aquaculture.
- Effective use of antibiotics for bacterial diseases relies on isolate specific antibiograms.
- Minimising antibiotic use in an aquaculture environment is required.

The antibiotics routinely tested for are:

- trimethoprim-sulfamethoxazole
- amoxycillin-clavulanic acid
- doxycycline
- erythromycin
- flumequine
- enrofloxacin
- neomycin
- oxytetracycline
- oxolinic acid
- minocycline
- gentamicin

A bacterial isolate is spread plated onto standard Mueller-Hinton agar and allowed to produce an even growth over the plate. Then, commercial antibiotic discs are dispensed onto the agar with a disc dispenser in equally spaced positions. The plate is incubated and zones of inhibition around the antibiotic discs are measured. An antibiotic is considered bacterially resistant when the zone diameters do not exceed specified limits based on published data for the antibiotic and its concentration in the disc (Fig. 92).

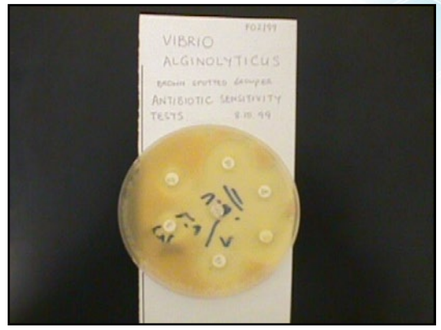


Figure 92. Antibiogram plate and discs.

Immunological Tests

Antibody-antigen tests are very useful to supplement cultural techniques for bacterial identification. This is particularly so for agents that are very slow growing or have multiple serotypes that are biochemically very similar. An example is the *Renibacterium salmoninarum* ELISA (Table 8).

Table 8. *Renibacterium salmoninarum* ELISA

Renibacterium salmoninarum
Enzyme Linked Immunosorbent Assay

Renibacterium salmoninarum Enzyme Linked Immunosorbent Assay (ELISA) is used to rapidly determine the presence of 57,000 Dalton antigen (p57) of *Renibacterium salmoninarum* in the tissue of fish.

A kidney tissue sample is obtained with a cotton swab. The antigen, if present in the tissue sample, is then allowed to diffuse into sample dilution buffer in tubes that have been coated with antibody specific to the Bacterial Kidney Disease (BKD) p57 antigen. The antigen is allowed to bind to the coated antibody for 1 hour at room temperature before the tube is washed to remove unbound material. A biotinylated monoclonal antibody (MAb) directed to a different portion of the BKD p57 antigen is then added to the tubes and the tubes are incubated at room temperature for 1 hour before being washed. Streptavidin linked to horseradish peroxidase (streptavidin-HRP) is then added to all of the tubes and the tubes are incubated at room temperature for 30 minutes. During this incubation period, the streptavidin protein moiety (linked to HRP) binds to the biotin ligand that is conjugated to the MAb. Unbound streptavidin-HRP is removed by washing prior to adding the substrate, hydrogen peroxide and the chromogen, ABTS.

If the test kidney tissue sample is from a fish that is infected with *Renibacterium salmoninarum*, a colour change will develop. This results because the MAb that is coated on the tubes captures the soluble BKD p57 antigen in the tissue sample. This allows the second biotinylated MAb to bind to the captured antigen. In turn, the streptavidin-HRP attaches to the biotin, which then reacts with the hydrogen peroxide/ABTS solution causing a green colour to develop. The colour change is proportional to the amount of antigen that is bound to the MAb.

If, on the other hand, the test sample is from a fish that is not infected with BKD and does not have antigen present in the kidney sample, no colour change will be observed. This results because there is no captured BKD antigen for the biotinylated monoclonal antibody to bind to and it is therefore removed by the washing step. Thus, there is no ligand for the streptavidin-enzyme complex to bind to and this complex is not present to react with the hydrogen peroxide and ABTS in the final step.

INTERPRETATION

Positive for infection with *Renibacterium salmoninarum*:

Those test samples yielding a colour change equal to or darker than the Low Positive Control should be considered positive for BKD. There is a rough correlation between the colour change and the level of infection. The darker the colour, the more antigen is present, and thus, the higher the level of infection.

Negative for infection with *Renibacterium salmoninarum*:

Those test samples resulting in a colour change less than the Low Positive Control should be considered negative for BKD by this ELISA.

Parasitology

The study of fish parasites is important because parasitism is very common in fish. In fact, it would be rare not to find a few parasites in every sample of fish. However, determining their pathogenic significance relies on adequate identification of the parasites. Methods for fish parasitology routinely used are:

- General necropsy examination
- Wet mount preparations
- Blood smear examinations
- Histopathology
- Preservation methods

As with any test, the quality of the sample for parasitology must be considered since poor samples do compromise the accuracy of any attempt to identify the parasite(s).
Table 9 details the value of various samples to parasitological investigations.

Table 9. Diagnostic usefulness of different tissue preservation techniques for identifying fish parasites^ (adapted from Noga (1996))

Specimen	Live fish	Dead fish#	Iced fish*	Frozen fish%	Fixed fish@
Protozoan ectoparasites!	+++	-	+	+	++
Monogenean ectoparasites!	+++	-	++	+	+
Metazoan parasites!	+++	++	+++	++	+
Myxozoa & Microsporea!	+++	++	+++	++	++

^ The ability to recover various parasites varies greatly; these comparisons are only intended as general guidelines. +++ = best, - = virtually useless.

! Comparisons between live, dead, iced and frozen fish are based on the ability to identify parasites in wet mounts; the diagnostic usefulness of fixed fish is based on the ability to identify parasites in histologic sections.

Dead fish left in the water at room temperature for 6-12 hours.

* Live fish immediately placed in a plastic bag after death, on wet ice for 6-12 hours.

% Live fish immediately placed in a plastic bag after death, frozen at –20 ºC.

@ Tissues from live fish immediately placed in 10% neutral buffered formalin.

General Necropsy Examination

Make a thorough, methodical examination of the fins, body, buccal cavity and gills. Carefully remove large ectoparasites and place them in a small container of the water used to submit the fish for later identification and/or preservation. Skin samples should be taken from several areas, including lesions, before excessive handling of the fish causes a loss of smaller, fragile ectoparasites. The use of anaesthetics may also cause some parasites to detach from their host. A blood sample can be collected for preparation of both wet and air-dried smears.

After a wet mount count of parasites on a gill biopsy, several gill arches can be excised, placed in water and gently shaken to dislodge small monogeneans, which may be found in the sediment. These are removed and placed in physiological saline for later fixation. Arthropods collected from the sediment can be placed directly in 70% ethanol, since they do not contract at death like many of the softer parasites.

Examine the internal organs. The abdominal cavity may contain larval digeneans, nematodes, cestodes or acanthocephalans. Larval helminths may be encysted in the viscera, and pseudocysts of microsporidians and myxozoans may be found in various organs.

Squash preparations of liver, gall bladder, spleen, urinary bladder, swim bladder, kidney, gonads and heart can be made. The gastrointestinal tract should be examined by opening the viscera with a pair of fine scissors and making wet mounts to look for adult worms and protozoa or coccidia.

Incisions should be made in the muscle to look for encysted parasites. The eyes should be examined carefully for larval digeneans in the lens and vitreous humour. Finally the brain should be removed and squashes made. It is also required to fix representative tissues for histopathology.

Wet Mount Preparations

Gill biopsy and wet mount examination

Immediately before biopsy the gills should be examined grossly. The visible gill filaments projecting caudally from the cartilaginous gill arch (4 pairs) are composed of a primary lamella providing support and vascular supply to half-disc-shaped secondary lamellae running transversely across it. Goblet (mucus) cells are numerous on the edges and tips of the primary lamellae.

Insert the tip of a pair of fine scissors under a number of gill filaments. Cut the tips of the filaments (do not include the cartilage arch unless the fish is very small <3 cm long). Place the biopsied filaments onto a glass slide, add 2-3 drops of water and cover with a cover slip. Spread out the filaments to minimise overlapping of filaments, as this will enable good visualisation and counting of parasites and filaments. Avoid having too many filaments on a single wet mount because bleeding from the filaments will obscure some smaller ectoprotozoa.

Gill parasites to look for are:

Trichodina, Chilodonella (Brooklynella-marine*), Dactylogyrus (Haliotrema*-marine gill flukes*), Ichthyophthirius multifiliis (Cryptocaryon irritans*-marine*), Myxosporeans, Oodinium (Amyloodinium*-marine*) and Costia,* now known as *Ichthyobodo* sp.

Gill parasite count estimate –

1. Fish have approximately 700 – 1,400 primary gill filaments. This is dependent on fish species, age and size of fish with filaments increasing as fish grow. Each gill arch (1, 2, 3 and 4) will have different numbers of filaments with the 2nd and 3rd arches having more filaments than the 1st and 4th arches. Also the anterior hemibranch has more filaments than the posterior hemibranch (Caltran and Silan, 1996; Dahal, 2003).

2. Count or estimate the number of gill flukes, *Trichodina*, *Brooklynella* or cystic structures (*Myxobolus*, *Epitheliocystis*).

3. Count the number of primary gill filaments on the wet mount.

4. Estimate the total number of gill parasites using 1,000 filaments as the total filaments per fish, and assuming that parasite distribution is consistent across all filaments =
   (Parasite count on wet mount/no. of primary gill filaments) X 1,000.

5. Classification of gill parasite load – refer to Table 10.

Table 10. Clinical significance of gill parasite number

Parasite Count/Fish	Loading	Potential Significance
0 - 200	Low	Low probability of impact on fish health.
200 - 400	Moderate	Stress on fish, gill damage beginning, reduced tolerance to bacterial infection and poor water quality. Treatment required.
400 - 600	Heavy	Stress on fish, gill hyperplasia and fusion. Very susceptible to respiratory, osmoregulatory and excretory failure. Bacterial infection very likely. Must treat to reduce loading.
600 – 800 or above	Extreme	Severe stress on fish, direct mortalities can occur due to gill failure. Gill necrosis, fusion and hyperplasia. Bacterial damage risk is high. Treatment itself can kill fish due to stress of handling and must be carried out very carefully with attention to aeration, +/- sedation.

Caveats:

1. The assessment of the clinical significance of gill parasite numbers in Table 12 is a general approach and important differences due to species, age of the fish, nutritional conditions, water quality conditions, husbandry methods and other concurrent diseases play a large role in the variability of responses of fish to parasite loading on the gills.

2. In general, the higher the number of parasites, the greater the risk of injury to gills, and the associated inflammatory reactions are likely to be more intense. With increased mucus production, hyperplasia and fusion can amplify gill dysfunction. As an example, grass carp (*Ctenopharyngodon idella*) with 30-100 *Dactylogyrus lamellatus* is considered a low infestation, diseased fish carrying 150-250 flukes could have 500-700 flukes after 2-5 months, resulting in fish mortalities (Molnár, 1971). In carp larger than 35 mm, intense infections even with 300+ parasites did not kill fish although there was gill damage localised at the apices of the gill filaments (Woo, 1999). A mixed infection of more than 1,000 *Pseudodactylogyrus bini* and *P. anguillae* in eels of 40 cm could not be conclusively implicated in eel mortalities (Woo, 1999). Our experience with marine grouper *Epinephelus lanceolatus* indicates that where an estimated 5,511 gill flukes/fish are present, they cause gill pallor (anaemia, excessive mucus and gill necrosis) which results in mortalities attributable to the flukes.

3. Some parasites will be more pathogenic than others because of their activities on gill tissue (crushing attachment, penetration, mobility, active consumption of gill tissue). This can result in greater injury to fish with relatively fewer parasites. The degree of attachment of the parasite to gill tissue may influence the impact, e.g., fewer parasites that are firmly attached may cause the same or greater damage than more parasites which are loosely adherent on the gills. Larger parasites may have a greater impact than small parasites.

4. Parasites that are well encapsulated, e.g., spores within plasmodia (myxozoan and microsporidians) may cause a space-occupying lesion on the gills. These do not move, not feed on the gill tissue but can cause structural alterations that impair gill function over time. Hence the number of plasmodia needs to be relatively high to inflict significant injury to the gills.

5. Multiple species of parasites may result in different gill injuries, which collectively can be more severe than single parasite species infestations.

Skin scraping and examination

Using a clean scalpel or glass cover slip (for very small fish), scrape the skin mucus including some scales in a head to tail direction. Scrape along the flank, behind the pectoral fins and along the edge of lesions. Place the scraping immediately onto a glass slide, add 2-3 drops of water and examine under a compound microscope without delay, as many protozoa will soon die after removal from their host. Many parasites are difficult to identify when dead. For very motile protozoa, use methylcellulose solution to slow them down if required. 40x to 100x magnifications are usually adequate for identifying most protozoa.

Skin parasites to look for are –

Trichodina, Chilodonella (Brooklynella-marine*), Gyrodactylus, Ichthyophthirius multifiliis (Cryptocaryon irritans*-marine*), Oodinium (Amyloodinium*-marine*), Costia (Ichthyobodo* sp.-marine*), Lernae* sp. (Anchor worm), *Benedenia* (marine)*, Trematode metacercaria* (the same as larval digenean flukes), *Argulus* (Fish louse), leeches, copepods and isopods.

Skin parasite count – do a total count of parasites on the skin scrape and note it as number of parasites per x40, or x100 field, e.g., a count of 50 Trichodinids at X40 field is equivalent to a count of 20 at x100 field. For large skin parasites, e.g., anchor worm and leeches count the number on the fish's body.

Intestinal scrape and smear

Cut a section of the small and large intestine. Open along the length of the gut. Scrape the lining of the gut onto a glass slide. Add 1-2 drops of water and examine for helminth or coccidia. Remove large nematodes using forceps. For faeces, use a thin smear of the contents, add 1-2 drops of water and use a cover slip for microscopic examination.

Gastrointestinal parasites to look for are – adult nematodes (round bodied worms, e.g., *Camallanus, Anisakis* and *Capillaria*), acanthocephalans (thorny-headed worms), and cestodes (tapeworms, e.g., *Bothriocephalus* sp.). Coccidial oocysts and trophozoites (e.g., *Eimeria, Goussia* and *Cryptosporidium*). Also note the presence of any motile flagellates such as *Hexamita.*

Organ or tissue squash preparations

It is often useful to make tissue squashes, e.g., for *Glugea* sp. in marine fish, of black xenoma cysts to confirm the morphology of spores. Squashes of the kidney, spleen, liver or any lesions can be made. Small fish can be squashed whole or the entire viscera removed and squashed. Cut a small piece of about 8 mm^3 of tissue, place it on a slide with a drop of water and squash with a cover slip. Examine under x100 or x400 looking for parasites, spores or granulomas.

Blood Smear and Examination

Blood can be taken from fish to look for haemoflagellates such as *Cryptobia, Trypanoplasma* or *Trypanosoma.* Heparinised syringes and blood tubes should be used. Blood is collected by venipuncture from the ventral spinal vessels, from the heart or from the gill vessels or the Duct of Cuvier in salmonids. A capillary tube placed on the cut tail stump can also draw sufficient blood for a smear. The smear should be thin enough and stained after being air-dried with Wright-Giemsa (Fig. 93).

	FISH BACTERIOLOGY WORK INSTRUCTION	FBW-2.3

WRIGHT STAIN

Staining procedure

1. Flood the smear with Methyl Alcohol.
2. Leave for one minute then pour off the Methly Alcohol and leave to dry.
3. Add 1.5ml "Eosin Methylene Blue Solution according to Wright (E4) (Fluka)" onto the smear.

CAUTION:	Eosin Methylene Blue Solution according to Wright:- Highly flammable and Toxic – Wear protective clothing (laboratory coat and gloves) when handling. Keep away from sources of ignition.

4. Leave for one minute.
5. Add 1.5ml pH 6.8 Buffer onto the smear.
6. Leave for seven minutes.
7. Pour off the solution and wash the smear with pH 6.8 Buffer.

Figure 93. Wright-Giemsa staining.

Histopathology

While fish parasites may be visualised in fresh preparations and these are extremely useful methods to see the whole parasite for identification purposes, histopathology is necessary to assess the pathological impact that these parasites may have on the host tissues (Figs. 94 & 95). The disadvantage of histopathology for parasite identification is that you only see two-dimensional sections of parasites, which can make it difficult unless you also have information from fresh preparations. Special staining such as Giemsa can be employed to highlight some parasites, for example, spores of microsporidians.

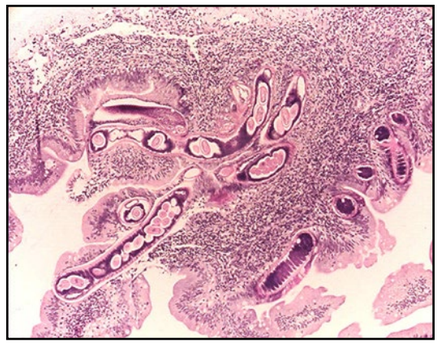


Figure 94. (H&E) Nematode larvae causing enteritis in discus (*Symphysodon discus*).

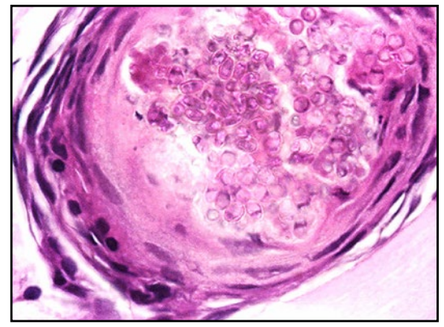


Figure 95. (H&E) Microsporidial spores in a granuloma; note the refractile nature of the spores.

Table 11. Recommended methods of preserving fish parasites (adapted from Stoskopf (1993) and Noga (1996))

Parasite group	Relaxation procedure	Fixation	Staining	Storage +/- Mounting
Monogeneans/ Digeneans^	Not needed for small worms. Gently flatten under cover slip and flood slide with fixative for 5 minutes.	Hot (55 °C-65 °C) AFA or hot NBF.	Hematoxylin aqueous	AFA or ETOH and mount in glycerol: ETOH
Cestodes^	Cold (4-8 °C) water or saline for 1-12 hours. Gently flatten under a cover slip and flood slide with fixative for 5 minutes.	Hot AFA, NBF or ETOH.	Hematoxylin aqueous	AFA or ETOH and mount in glycerol: ETOH
Nematodes*	Stretch large worms by each end on forceps. Add fixative for 5 minutes.	Hot AFA or ETOH	Hematoxylin aqueous	Small nematodes are cleared in glycerol: ETOH and mounted permanently in glycerol jelly. Large nematodes are cleared and temporarily mounted in glycerol: ETOH.
Acanthocephalans	Cold (4-8 °C) water or saline for 1-12 hours.	Hot AFA, NBF or ETOH. Puncture cuticle.	Hematoxylin aqueous	Small – stained. Large – unstained. Stored in AFA or ETOH. Mounted in glycerol: ETOH.
Arthropods	Not required	Cold (4-8C) ETOH.	Unstained	Cleared in 10% KOH or Hoyer mounting medium
Ciliates	N/A	Air dry smear^	Wrights giemsa. Stain immediately.	Add cover slip and Permount medium.
Flagellates	N/A	Air dry smear^	Wrights giemsa. Stain immediately.	Add cover slip and Permount medium.
Amoebae	N/A	Air dry smear^	Wrights giemsa. Stain immediately.	Add cover slip and Permount medium.
Myxozoa	N/A	Air dry smear^	Wrights giemsa. Stain immediately.	Add cover slip and Permount medium.
Microsporidians	N/A	Air dry smear^	Wrights giemsa. Stain immediately.	Add cover slip and Permount medium.

AFA = alcohol-formalin-acetic acid (Conc. Formalin 10ml, Acetic Acid Conc. 5 ml, ETOH 85 ml).
NBF = 10% neutral buffered formalin
ETOH = 70% ethanol
Glycerol: ETOH = 1 parts glycerol : 9 parts 70% ethanol
* Before beginning preservation procedures, encapsulated larvae should be manually dissected out of the capsule or the capsule should be digested with 0.2% pepsin in 0.1 M HCl.
^ Note that this method is less reliable for protozoan identification than routine histopathology but can be useful when submitting specimens to reference laboratories for identification.

Preservation Methods

Fixation and staining of parasites for future identification or archiving

Table 11 provides guidelines on the preservation of fish parasites. This process is time-consuming and is not required for commonly identified specimens. However new unknown species should be preserved for transfer to a reference parasitology laboratory for definitive identification should the need arise. For legal cases, e.g., food complaint cases from the Food and Environmental Hygiene Department (FEHD), specimens of parasites (including the fish specimen) must be labelled and stored. These samples will be surrendered to the FEHD.

Histological Techniques

Histology is the fundamental technique for understanding disease processes in fish health diagnosis and management. Its usefulness is as follows:

- Assessment of the role of pathogens in host tissues.
- Identification of emerging pathogens for which specific molecular or cultural techniques are not available.
- Assessment of host tissue changes due to non-infectious disorders.

However histology has the following limitations, which must be kept in mind by the pathologist:

- Susceptibility to artifactual changes which can cause misinterpretation of disease processes.
- Cannot in most cases differentiate to species level the identity of pathogens.
- Labour-intensive and time-consuming as a tool in health screening, lack of sensitivity when detecting disease agents of low prevalence.

Aspects of histological methods include:

- Tissue Fixation
- Cassetting (cutting in)
- Decalcification
- Processing & Embedding
- Sectioning, Mounting & Staining
- Special Stains

Tissue Fixation

Fish once dead decompose very rapidly, particularly the gills and gut. Therefore fish must generally be submitted live to the laboratory or as fixed specimens if useful histopathology is to be possible. Table 12 details the appropriate euthanasia and fixation methods.

Table 12. Fixation methods

Finfish	• 10% neutral buffered formalin, 10:1 volume fixative to tissue volume. • Humane euthanasia with (Tricaine Methane Sulphonate) MS222 and spinal cord severance. • Remove one gill cover and slit open the abdominal wall at ventral midline or remove one flank of the abdominal wall to allow adequate penetration of fixative to internal tissues. • For marine fish, 10% seawater formalin may be used to reduce osmotic induced artifacts if fixed in freshwater formalin.
Crustacea	• Davidson's solution injected directly into internal tissue through the shell of a freshly and humanely killed fish with (Tricaine Methane Sulphonate) MS222 or chilled in a fridge for 5 minutes. • Very small shrimp may be placed directly into Davidson's solution after humane euthanasia with MS222 or chilled in a fridge for 5 minutes. • 10:1 volume fixative to tissue volume.
Mollusc	• Humane euthanasia by chilling in a fridge for 5 minutes. • Deshell and fix soft viscera in 10% neutral buffered formalin, 10:1 volume fixative to tissue volume.

Cassetting (cutting in)

There are no hard and fast rules for cutting fish tissues into histology cassettes, but the guidelines in Table 13 will enable consistent orientation and presentation of the major tissues to make it easier for interpretation of histopathology.

Table 13. Tissue orientation in histology cassettes

Gills	• Lay the gill arches flat and together in one cassette. • This will enable even sectioning; otherwise if various tissues of differing thickness, e.g., liver are placed with the gills, then the thinner tissues may be missed by the sectioning while trying to capture the full section of the thicker tissues. • This also enables segregation of tissues that need decalcification from those that do not.
Skin and muscle	• A slab of skin with the plane of sectioning across the various layers of the skin should be flat and placed in the cassette. In other words, don't place the scaled surface on the cassette.
Liver and spleen	• Cut a transverse section through these organs and place the flat surface on the cassette; avoid having the rounded edges presented on the cassette.
Intestines and stomach	• Both longitudinal and transverse sections should be included with the flat edge of the tissues lying face down on the cassette.
Eye	• Section the eyeball through the midline; include both halves as usually only one half will have the lens.
Heart	• Section longitudinally through the sinus venosus, atrium, ventricle and bulbus arteriosus and place the flat edge on the cassette.
Brain and spinal cord	• Section longitudinally; place the flat edge on the cassette.
Kidney and vertebral column	• Section transversely; place the flat edge on the cassette.
Whole shrimps	• Section longitudinally; place the flat edge on the cassette.

Decalcification

Decalcification removes the hard calcium from the tissues, making them soft enough for thin sectioning. The following tissues require decalcification prior to embedding.

- Gills
- Skin
- Kidney with spinal column
- Bone
- Shell of crustacea or molluscs

The following methods of decalcification may be employed:

- Dilute acid (hydrochloric acid, picric acid (Bouin's solution).
- EDTA
- Davidson's solution

The key is not to over decalcify as this will damage the soft tissue cellular structures. Under-decalcification will make sectioning difficult, resulting in fragmentation of the thin section. The duration of decalcification controls the degree of decalcification.

- Hydrochloric acid: 2-3 hours depending on strength
- Picric acid (Bouin's solution): 12 hours
- EDTA: 2-3 days
- Davidson's solution: 24 hours

Processing and Embedding

This is a standard method for all tissues whereby wax is used to hold the tissues in a block after the tissues have been completely dehydrated having passed through a series of alcohol baths over several hours in an automated processor. It is important to work with the histologists to ensure that the orientation of tissues in the cassette reflects what the fish pathologists require. This would also apply to the decalcification process in order to minimise tissue artifacts.

Sectioning, Mounting and Staining

Standard histological methods apply to fish tissues, whereby 4-5 μm thin sections are sliced from the wax block after hardening on a freezer plate. At this stage, adequate decalcification of hard tissues and removal of all moisture from tissues is critical for producing good-quality thin sections that do not fragment or crimple.

The thin section is floated on a hot (40 ^{0}C) water in which the wax is melted and the tissue is picked up on a glass slide. This is then stained with Haematoxylin and Eosin (H&E) after which the slides are mounted with coverslips using a mounting medium. After a period of drying and labelling, the H&E slides are ready to be examined by the pathologist using a light microscope.

Special Stains

Special stains may be applied to recuts of sections in relation to which the pathologists are interested to differentiate tissue changes or to identify the presence of pathogens and abiotic substances (Table 14).

Table 14. Histological special stains

Special stain	Function/Stain reaction
Giemsa	Identify parasites, e.g., microsporidians, metaceracerial cysts/stains blue.
Periodic Acid-Schiff (PAS)	Identify glycogen/stains deep pink.
Gram	Identify bacteria/Gram-negative (red/pink) and Gram-positive (blue/purple).
Ziehl-Neelsen (ZN)	Identify acid-fast bacteria, e.g., Mycobacteria and Nocardia spp./ stain pink-red. Identify liver ceroid/stain pink-red.
Perl's Prussian Blue	Identify iron and iron containing haemosiderin/ stains blue.
Fuelgen	Identify DNA, particularly viral DNA of intracytoplasmic viruses/DNA stains red.
Sudan Black	Identify lipids/stains black (only on frozen sections). Also stains chitin.
Oil-Red-O	Identify lipids/stains red-orange (only on frozen sections).

Virology

Clinical virology is an essential discipline in the diagnosis of infectious viral diseases in fish. It includes the following aspects:

- Cell culture
- Immunological methods
- Electronmicroscopy
- Molecular methods

Cell Culture

Since viruses require living cells to replicate, in order to isolate enough viruses for identification tests, cell lines are needed for sample inoculation. This discipline consists of the development of fish cell lines from primary cells and tissues to the stage where they become 'self-perpetuating'. The cells are then maintained in a condition where they remain viable and susceptible or sensitive to specific viral inoculations. The inoculation of fish cell lines with samples for which viruses need to be screened is as much an art as it is a science. This is because each virus and each cell line have their own unique host-pathogen interactions.

For new viral diseases, primary cell line development may be necessary if existing commercial fish cell lines are not sensitive to the new virus. Here, usually the target tissue and cells are used to assess the nutrient conditions needed to maintain viability and sensitivity. Multiple passages are required to build up a stockpile of healthy cells. Depending on the cell type, passages of the order of 200-300 may be required to establish the suitability of a cell line as being 'immortal'. This activity is really in the research realm and for most routine diagnostic laboratories, commercial fish cell lines are purchased to begin fish virology work (Table 15). The nutrient media, incubation temperature, control of microbial and fungal contamination and sub-passaging are necessary routine work to maintain a viable set of fish cell lines (Fig. 96). This is labour-intensive and requires considerable attention to aseptic techniques (Table 16), laboratory work space, expensive reagents, nutrient media (Table 17) and incubators.

Table 15. Fish cell lines used in AFCD

Fish cell line	Viruses detection
Epithelioma papillo-ma cyprinum (EPC)	Spring viraemia of carp virus (SVCV) Infectious pancreatic necro-sis virus (IPNV) Viral haemorrhagic septicae-mia virus (VHSV) Infectious haematopoeitic necrosis virus (IHNV)
Fathead Minnow (FHM)	SVCV, IPNV
Chinook Salmon Em-bryo (CHSE-214)	INPV
Bluegill Fry (BF2)	IPNV, IHNV, VHSV
Striped Snakehead (SSN-1)	Nodavirus
SeaBass (SB)	Nodavirus

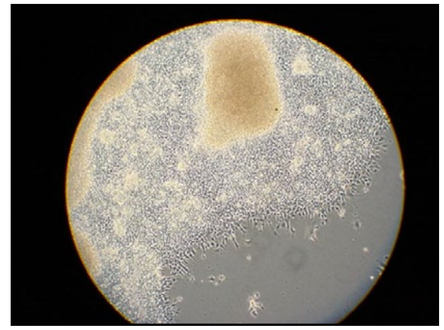


Figure 96. EPC cells becoming confluent on a culture plate.

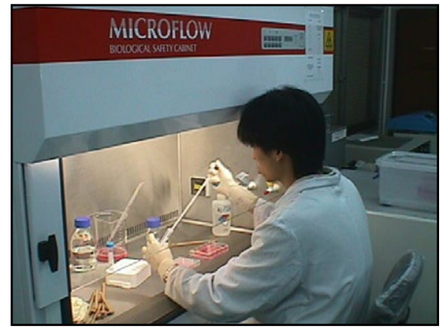


Figure 97. Working with cell cultures in a biological safety cabinet.

Table 16. Asepsis in virology

General Disinfection:

1. Keep doors and windows closed during the laboratory session to prevent contamination from air currents.
2. Wash hands with liquid detergent and dry them with paper towels upon entering and prior to leaving the laboratory.
3. Tie back long hair to minimise hair contamination of samples or reagents.
4. Wear a lab coat for the tissue culture room or apron while working in the tissue culture room.
5. Closed shoes should be worn at all times in the laboratory
6. Do not smoke, eat or drink in the laboratory.
7. Disinfect the working area before and after testing procedures.
8. Keep the UV light on when the laminar biosafety cabinet is not in use.
9. Wipe the bench with 200ppm chlorine or 2% bleach before and after any virology work. (Caution: do not apply bleach on metal (as it will cause corrosion) or polystyrene (as it will damage it) surfaces.)
10. Perform cell culture daily maintenance work first, and perform virology work later.
11. Turn on the UV lamp after performing any virology work in the cabinet.

Contaminated, infected materials:

1. Discard waste into the beaker containing 200ppm chlorine as disinfectant.
2. The contaminated materials must be collected in a high temperature-tolerant polypropylene biohazard bag for disinfection by autoclaving.

TISSUE CULTURE ASEPTIC TECHNIQUE

General:

1. Wipe the Biosafety Cabinet with 70% alcohol before and after use.
2. Wear gloves during handling cell culture materials and preparing medium.
3. Make sure that all the materials applied in cell culture are sterilised.
4. Wipe test consumables with 70% alcohol before being placed into the Biosafety Cabinet.
5. Do not allow the tissue culture materials to touch any contaminated surface during the procedure.
6. Aseptic technique on handling liquids in the Biosafety Cabinet:
   - 6.1 Avoid fomite contamination of bottled liquids when using them.
   - 6.2 Pipetting – do not allow the pipette to touch the bottle.
   - 6.3 Do not put a pipette down and reuse it.
7. Cover the lips of flasks, bottles or special tubes as often as possible or seal with parafilm.
8. Remove all excess fluids using an aseptic technique and dispose of in a disinfectant container.
9. Dispose of broken glass and needles into the sharps box.

Control of contamination sources:

Contamination in the cultivation of the cell lines must be avoided. If contamination occurs, e.g., 25% of cell-line wells, the following items should be checked:

1. Instrument: Biosafety cabinet is in good working condition. Bottles and materials are sterilised. Incubators are in clean condition.
2. Reagent/Media: Packaging is in good condition. Not expired. The concentrations of antibiotics used are appropriate.
3. Environment: The floors, walls, ceiling and exhaust system are regularly cleaned of dust, etc.
4. Staff: Use clean laboratory coats. Clean hands or wear new gloves during lab work. Clean shoe soles before entering the laboratory.

Table 17. Virology media and reagents

CELL CULTURE MEDIUM PREPARATION

Phosphate Buffered Saline without potassium and calcium (PBS)

- Dilute the 10X PBS (Gibco^{R}) stock solution with autoclaved Milli-Q^{R} waterl.
- Autoclave the diluted PBS.
- Storage period: 6 months at romm temperature.

4.4%(w/v) Sodium bicarbonate solution

- Weigh 0.1 g of phenol red and 44 g of sodium bicarbonate.
- Dissolve in 1000 ml of distilled water and carry out autoclave sterilisation.

Gentamicin (100x) stock solution (Stock: 5 mg/ml; working: 50 µg/ml)

- Weigh 0.7836 g of Gentamicin powder (Sigma^{R}, 638 µg of Gentamicin base/mg).
- Dissolve in 100 ml dH_2O and sterilise the stock solution by filtration through 0.22 µm filters.
- Storage period: 6 months at –20 °C.

Freezing cocktail

- Mix 10 ml of Foetal Bovine Serum (GibcoR), 10 ml of DMSO (SigmaR) and 80 ml of antibiotic free medium.
- The reconstitution process should be conducted in the biosafety cabinet. Storage period: 3 months at -20 °C.

Transport medium

- Measure 25 ml Hank's balance salt (10X) stock solution.
- Add 12.5 ml antibiotic (100X) stock solution (Gibco^{R}/ Sigma^{R}).
- Add 5 ml Fetal Bovine Serum (GibcoR). Add 5 ml 4.4% Sodium Bicarbonate.
- Fill up to 200 ml by using Milli-QR water. Adjust pH to pH 7.2-7.4 by using HCl.
- Fill up to 250 ml by using Milli-QR water. Perform vacuum filtration of constituted media. Storage period: 1 month at 4 °C.

Trypan Blue 0.4% (w/v)

- Measure 0.4 g of Trypan Blue in 100 ml of PBS. Filter through 0.45 µm filter paper.
- Storage period: 1 year at room temperature in screw cap bottles. Filter before use.

MEM Base

- Dissolve a package of MEM powder (Gibco^{R}) into 900 ml of autoclaved Milli-Q^{R} water
- Add 12.5 ml of 26.67% NaCl.
  16 ml of 4.4% $NaHCO_3$.
  5 ml of 1 M hepes (Gibco^{R}).
- Adjust pH to 7.2 – 7.4 by using 2% HCl. Fill up to 1 L with autoclaved Milli-Q water. Perform vacuum filtration of the constituted media. Label. Storage period: 3 months at 4 °C.

DISINFECTANT

1% Virkon^{R} for the disinfection

- Dissolve 1 packet of Virkon^{R} powder into 2.5 L of water by stirring to form a clear pink solution. Fresh preparation is better.
- Storage period: 7 days at room temperature in a brown bottle (to protect it from direct light).
- **Caution with the Virkon powder**: It is irritating to the skin, do not breathe in the dust, and avoid contact with skin and eyes.

1000 ppm chlorine stock solution

- Dissolve 1 chlorine release tablet (Haz-Tabs, Guest Medical, Cat no: H8801) into 500 ml of water. Dilute five-fold before use.
- **Caution:** Contact with acids liberates toxic gas. It is harmful if swallowed. It is irritating to the eyes and respiratory system. Contact with combustible material may cause fire. Refer to the materials safety data sheet (MSDS).

MEM Base

- Dissolve a package of MEM powder (Gibco^{R}) in 500 ml of Autoclaved Milli-Q^{R} water.
- Add 45.5 ml of 4.4% sodium bicarbonate stock solution.
- Fill up to 900 ml with autoclaved Milli-Q^{R} water. Adjust pH to pH 7.2 -7.4 by using 2% HCL.
- Fill up to 1 L with autoclaved Milli-Q^{R} water.
- Filter and sterilise.
- Storage period: 3 months at 4 ºC.

L15 Base

- Dissolve a package of L-15 medium powder (Gibco^{R}) in 500 ml of autoclaved Milli-Q^{R} water.
- Add 45.5 ml of 4.4% sodium bicarbonate stock solution and 5 ml of HEPES Buffer.
- Fill up to 1 L by autoclaved Milli-Q^{R} water.
- Adjust pH to pH 7.2- 7.4 by using 2% HCl.
- Vacuum filtrate the constituted media.
- Storage period: 3 months at 4 ºC.

Complete culture medium

- Mix 450 ml of basic culture medium with 50 ml of Foetal Bovine Serum (FBS) (Gibco^{R}) and 5 ml of antibiotics in the biosafety cabinet
- Remark: The type of antibiotics should be changed every two months.
- Storage period: 1 month at 4 ºC.

Antibiotic free medium

- Add 50 ml of Foetal Bovine Serum (Gibco^{R}) to 450 ml of basic medium and mix well.
- The reconstitution process should be done in the biosafety cabinet.
- Storage period: 3 months at 4 ºC.

2 % culture medium

- 4 ml of Foetal Bovine Serum (Gibco^{R}) and 2 ml of antibiotics mix with 194 ml of basic medium.
- The reconstitution process should be conducted in the biosafety cabinet.

A set of viable cells are prepared by retrieving nitrogen frozen cells from their frozen state (Table 18) and inoculating these into culture plates or flasks (Table 19).

Preparation of tissue samples for inoculation involves the following steps carried out in a biological safety cabinet (Fig. 97):

- Target organs for virus culture depending on the disease of interest, but usually include gills, spleen, kidney, heart and brain.
- The tissues are held in transport media and homogenised.
- The material is filtered and supernatant is collected for inoculation onto confluent cell cultures (Table 21) which have been set in 24-well plates (Table 20, Fig. 101).
- Virus viability is influenced by the freshness of the samples, the number of freeze-thaw cycles the sample may have been subjected to and freedom from chemical preservatives, e.g., formalin.

Table 18. Retrieving cells

EQUIPMENT

1. Liquid Nitrogen Storage System (LNSS) (ThermolyneR, Locator, Cryo Biological Storage SystemR)
2. Frozen cell line storage book
3. Cell lines in liquid nitrogen tank
4. 37 °C water bath
5. Class II Biosafety Cabinet
6. Sterile pipettes and pipette aid
7. Media
8. Foetal bovine serum
9. Sterile 25 cm^2 tissue culture flasks
10. Sterile 15 ml centrifuge tube
11. Tabletop centrifuge
12. CO_2 incubator
13. Waste bottle containing disinfectant
14. Inverted microscope in cell culture laboratory

METHOD

Caution: when handling DMSO, always wear gloves and work away from fire.

1. Find out the location of the cell line from the frozen cell line storage book.
2. Go to the LNSS. Use the protective gloves to take out the required cryopreserved vial(s) and place the vial(s) in the water bath at 37 ºC.
3. Pour the content of the vials into the centrifuge tube and centrifuge at ~80 g in the tabletop centrifuge for 10 min to pellet cells.
4. Discard the supernatant into the waste bottle and resuspend cells in ~10 mL of complete media and place into a 25 ml culture flask.
5. Label the flask with cell type, date and passage number.
6. Update the records in the frozen cell line storage book (in the cell culture laboratory).
7. Place the flask into a cell culture incubator.
8. Examine cells daily.

Table 19. Cell line maintenance techniques

A. DAILY OBSERVATION OF CELL LINE AND MEDIUM

1. Wipe the table and the microscope with 70% alcohol.
2. Check the clarity of the media. If it is turbid, it is considered to be contaminated and should be discarded.
3. Check the colour of the medium. If the medium is yellow, it shows that the medium is of low pH. Check for bacterial contamination of the medium with a microscope at 40X objective. If there is contamination, it should be discarded. If there is no contamination, change to fresh medium.
4. Check the date of the culture flask. Media should be replaced as required. This ensures effective antibiotic activity in the medium.
5. Observe the flask with low power microscopy to assess the confluency of cells over the substrate surface. Then observe the flask with high power to check the bacterial contamination. If there is 90-100% confluency of cells, the flask should be sub-cultured.

B. SUB-CULTURE TECHNIQUE (TRYPSINIZATION)

1. The process is carried out in the bio-safety cabinet.
2. Place the waste bottle containing disinfectant into the cabinet.
3. Wipe the flask where the cells are 90-100% confluent with 70% alcohol and put the flask into the cabinet.
4. Discard the old medium of the flask.
5. Add sterile PBS into the flask.
6. 25 cm^2 flask: 2-5 ml; 75 cm^2 flask: 5-10 ml; 125 cm^2 flask: 10-15 ml.
7. Rinse the monolayer of cells with PBS very gently.
8. Discard the PBS.
9. Repeat steps 5 to 7 to wash again.
10. Serum in the medium can inhibit the function of trypsin. This is the reason why the washing step is needed before trypsinisation.
11. Add sterile 0.25% Trypsin-EDTA into the flask.
12. 25cm^2 flask: 0.5ml; 75cm^2 flask: 1.5ml; 125cm^2 flask: 2.5ml.
13. Tilt the flask to spread trypsin evenly over the monolayer of cells.
14. When the cells begin to loosen, tap the flask against the palm of your hand. Add fresh complete medium into the flask. Break up the clumps of cells by repeated pipetting of the cell suspension within the pipette
15. Discard the excess suspension into the waste bottle or into a new sterile culture flask for the new culture.
16. Label the passage number and the date on the flask.
17. Caution: If the flask has been used for eight passages or more, the flask must be discarded. The cell line should grow in a new culture flask.

Table 20. Cell plates preparation

PROCEDURE

A. CHOOSE A CONFLUENT FLASK

1. Wipe the bench and the microscope with 70% alcohol.
2. Take out the cell culture flask from the CO_2 incubator wearing gloves and a lab coat.
3. Observe the growth of cells under a microscope at lowest power (10X).
4. Choose the flask with a confluent cell sheet to provide cells in the 24-well plate. (Two 25 cm^2 flasks for one 24-well plate; two 75 cm^2 flasks for three 24-well plates).

B. SEED CELLS ONTO THE 24-WELL PLATE

1. Use aseptic techniques.
2. Spray the external surfaces of flask(s), sterile 24-well culture plate(s), 10% MEM flask, trypsin-EDTA and sterile pipette(s) with 70% alcohol. Place the materials into the biosafety cabinet.
3. Spray the waste bottle containing 200 ppm chlorine with 70% alcohol and place it into the biosafety cabinet.
4. Trypsinise the cells of the flask.
5. After trypsinisation and suspension of the cells, open the package of the sterile 24-well plate(s) and add 1 ml of cell suspension into each well of the culture plate(s).
6. Label the cell line, passage number and date on the edge of the culture plate.
7. Update the cell line maintenance record book.
8. Wrap the plate with parafilm and incubate in the CO_2 incubator overnight.

C. CHANGE 2% MEDIUM BEFORE INOCULATION

1. Spread the 24-well culture plate(s) with 24-hour monolayer cells.Disinfect the outer surfaces of the cell plates with 70% alcohol and place it into the safety cabinet.
2. Disinfect the flask of 2% MEM, multi-dispenser, the disposal sterile pipette syringe package and waste bottle containing disinfectant with 70% alcohol, and place these into the cabinet.
3. Pipet the old medium out of the culture plate and discard in the waste bottle.
4. Add 1 ml of 2% MEM into each well by using a multi-dispenser.
5. The plate(s) will now be ready for inoculation.

Table 21. Virus sample inoculation

SIGNIFICANCE

Fish cells are used for targeted viral culture multiplying.

SPECIMEN (SCOPE AND APPLICATION)

Scope

The specimens are submitted as pooled kidney or spleen tissues in transport media. For fish gills or intestines, which may have bacterial contamination, stand them for at least 1 hour at room temperature or overnight at 4 °C in the antibacterial transport media prior to sample preparation. All procedures associated with virus isolation are conducted using aseptic techniques and performed in the biosafety cabinet.

Sample preparation

Specimen tissues are homogenised with a tissue homogeniser in 15 ml of transport as a 10% suspension.

The homogenised tissue is then clarified by centrifugation in the IEC cooled centrifuge at 2000 rpm for 10 min. The supernatant is collected and used as the inoculum. These suspensions are stored in four 2 ml frozen aliquots prior to testing.

QUALITY CONTROL

Un-inoculated wells included in every plate run parallel with the method serve as a negative control.

PRINCIPLE

The specimen suspensions are inoculated onto confluent cell sheets. The appropriate cell line for isolation is used based on it being sensitive to the suspect virus or virus to be screened. The presence of virus is determined by the observation of cell pathology or cell death (cytopathic effect or CPE).

REAGENTS

1. Confluent growth of a monolayer of cell line on a 24-well tissue culture plate
2. Inoculum
3. 2% culture medium

EQUIPMENT

1. 5 ml sterilised syringe
2. 0.45 um sterilised syringe filter
3. Class II Biosafety Cabinet
4. CO_2 incubator
5. Timers
6. Pipette aid
7. Inverted microscope
8. Deep Freezer

PROCEDURE

1. Defrost the inoculum to room temperature.
2. Use a 5 ml sterilised syringe to draw the 2% medium up to the 2.7 ml mark (prepare an adequate number of syringes before commencing).
3. Then draw another 0.3 ml of the specimen extracts up to the 3 ml mark of the syringe (final dilution of the tissue homogenate is 1:100).
4. Prepare the 24-well tissue culture plate by adding 1 ml of 2% culture medium to each well.
5. Use a 0.45 µm sterilised syringe filter to add 1 ml of inoculum into the culture well.

   1 drop into the first row of wells (2000x dilution), **2** drops into the second row (1000x dilution), **5** drops into the third row (400x dilution) and **10** drops into the fourth row (200x dilution).
6. Repeat steps (2)-(5) for subsequent specimen inoculation for the second line of wells until the second last line of wells of the culture plate.
7. Add 2% culture medium to the last line of wells of the plate as negative control well.
8. Label the plate with plate number.
9. Incubate at 15 °C for 1 hour.
10. Return to the 15 °C incubator for further incubation.
11. Cells are incubated for 7 days with daily observation (3-7 days) for CPE.
12. After 7 days in the absence of a virus-specific effect on the cells, the culture is passaged onto another plate. Transfer 200 µL of each well onto 1 ml of freshly monolayered cells and incubate for a further 7 days. A minimum of one passage is required before a sample is declared negative for virus.

FURTHER INVESTIGATION PROCEDURES

- ELISA antigen detection or PCR tests where CPE is observed to confirm virus identity
- Samples may also be sent to external reference laboratories for confirmatory testing to ensure that in-house results are correct.

RESULTS

Cytopathic viruses will produce changes to the cell monolayer, which can be detected by examination of the cell monolayer under an inverted microscope. The normal cell morphology and/or aging effects can be determined by comparison with the un-inoculated cell controls. The culture is considered negative in the absence of such changes after one sequential passage.

VALIDATION

Information on the diagnostic procedures for important viral pathogens such as IPN, IHN, VHS and SVCV are given in the OIE Diagnostic Manual for Aquatic Animal Diseases.

Once the samples have been inoculated onto confluent cell plates, the task for the virologist or laboratory technician is to make 24 hourly observations of the cells (Table 22). Timing of the observations is important for these reasons:

- Virus-induced cytopathic effects (CPEs) (Figs. 98-100) are relatively specific for viruses and the onset of occurrence is variable.
- Cytotoxic effects (CTEs) must be distinguished from CPEs and measures taken to control CTEs.
- CPEs are dynamic events which can be missed if regular observations are not carried out.

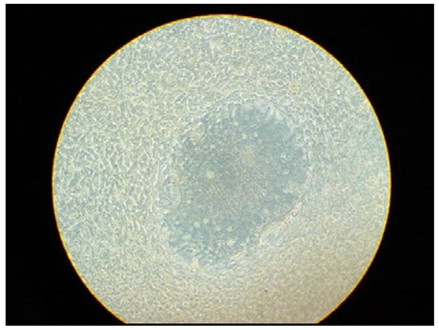


Figure 98. Developing CPE on a cell sheet.

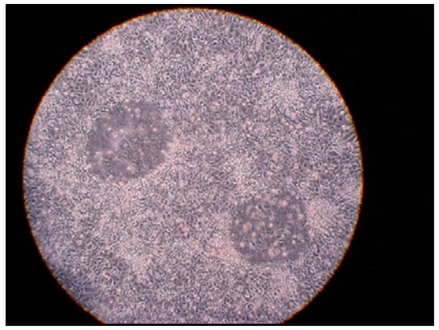


Figure 99. Spreading CPEs indicative of virus activity on cell culture.

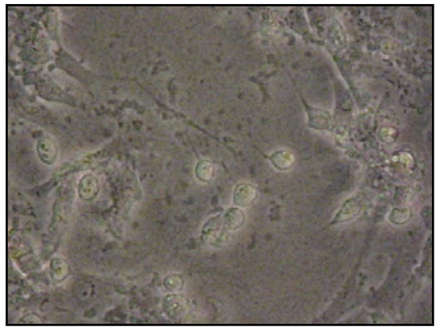


Figure 100. Rounding of cells adjacent to the edge of the CPE area.

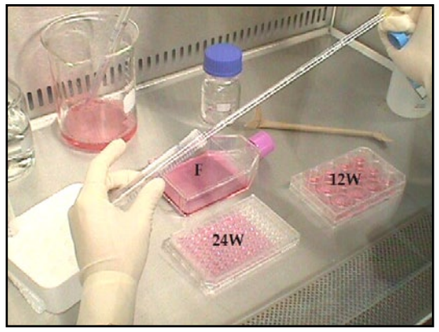


Figure 101. Flask (F), 12-well (12w) and 24-well (24w) plates used for virus inoculation.

Once cells are produced in excess of requirements, for long-term storage and to maintain a bank of available cell lines, these cells must be frozen into liquid nitrogen (Table 23). These cells need to be periodically thawed and assessed for their viability and sensitivity to viral infection as a quality assurance check for test results based on cell culture inoculation.

Table 22. Observation of inoculated samples

PROCEDURE

1. Wipe the bench and microscope with 70% alcohol.
2. Take out the inoculated 24-well plate(s) from the ‘sample incubation’ incubator wearing gloves and lab coats.
3. Observe the negative control column under the lowest power (10X). If there is any cytopathic effect (CPE) or any cytotoxic effect (CTE) on the negative control, the test will be repeated with a new plate.
4. If the cell sheet of the negative control is normal, observe the sample inoculated wells.
5. If there is CPE in the sample wells, the supernatant will be collected and stored at -70 °C. Virus-specific ELISA tests will be performed on wells with CPE and also the negative control well.
6. The appearance of CPE can include: syncytium formation (fusion of several cells to form a large multinucleate cell), refractile inclusion bodies in nuclei, giant cells formation, granular cells or clumped cells. There may be loss of confluency of the cell sheet and appearance of gaps, which may be circular or punctate.
7. Record the result of each well.
8. After observation, place the plate into the ‘sample incubation’ incubator and wipe the microscope and bench area with 70% alcohol.
9. The inoculated plate will be observed until 7 days after both initial inoculation and first passage.

Table 23. Preparation of cell lines for liquid nitrogen storage

The cell lines are cryopreserved to avoid loss by contamination, to minimise genetic change in continuous cell lines, and to avoid aging and transformation in finite cell lines. Before cryopreserving, cells should be characterised and checked for contamination.

EQUIPMENT AND MATERIALS

1. Liquid Nitrogen Storage System
2. Frozen cell line storage book
3. 37 ºC water bath
4. Class II biosafety cabinet
5. Sterile pipettes and pipette aid
6. Media
7. Sterile 25 cm^2 tissue culture flasks
8. Sterile 15 ml centrifuge tube
9. Tabletop centrifuge
10. CO_2 incubator
11. Waste bottle containing disinfectant
12. Inverted microscope in the cell culture laboratory
13. 2 ml plastic cryogenic vials
14. -70 ºC freezer

PROCEDURE

1. Choose the live fish cells for storage.
2. Discard the old medium and sub-culture cells using antibiotic-free medium.
3. Ensure that the cells are not contaminated with bacteria using antibiotics.
4. The flask(s) should be incubated until 90-100% confluence with cells in the CO_2 incubator.
5. Warm the freezing cocktail in the 37 ºC water bath.
6. Trypsinise the flask(s) of cells that are prepared for frozen storage.
7. Pipet the cell suspension into the sterile 15 ml centrifuge tube.
8. Centrifuge the tube at 800-1000 rpm for 10 min.
9. Discard the supernatant and resuspend the cell pellet into the freezing cocktail.
10. Three 25 cm^2 flasks – 2 ml freezing cocktail (1 ml per vial).
11. One 75 cm^2 flask – 2 ml freezing cocktail (1 ml per vial).
12. Pipet 1 ml of cell suspension with the freezing cocktail into each sterile cryogenic vial.
13. Screw the cap of the cryogenic vial tightly.
14. Label each flask with cell type, date and passage number on the vial(s).
15. Disinfect the external surface of the vial(s) with 70% alcohol and seal with parafilm.
16. Place the vial(s) into a 4 ºC refrigerator for 4 hours.
17. After 4 hours, remove the vial(s) into a 20 ºC freezer overnight.
18. Put the vial(s) into a pre-cooled transporting container with an ice pack and transfer the vial(s) into the –70 ºC freezer.
19. Freeze the vial(s) at –70 ºC freezer overnight.
20. On the next day, put the vial(s) into a pre-cooled transporting container with an ice pack and transfer the vial(s) into the cell culture room quickly.
21. Find the location where that cell type should be stored from the frozen cell line storage book.
22. Wear protective gloves and then put the cryopreserved vial(s) into the liquid nitrogen tank.

    Caution: When picking up the tray of the liquid nitrogen tank, it should be done gently to prevent the liquid nitrogen spilling out, which can be injurious upon skin contact.
23. Record the cell line storage book with date and cell type.

Immunological Methods

While the cell culture enables isolation of viable virions, it does not provide definitive identification of viral pathogens unless immunological methods (and PCR) are applied to the virus-containing supernatant. Such methods include Enzyme-linked Immunosorbent Assay (ELISA) employing specific antibodies of target viral pathogens. ELISAs are applied to cell cultures with demonstrated CPE effects. This is applied for the following diseases:

- Spring Viraemia of Carp
- Infectious Pancreatic Necrosis
- Viral Haemorrhagic Septicaemia
- Infectious Haematopoeitic Necrosis

An example of the work instruction to run an ELISA is provided as follows.

Immunoenzyme Assay Detection for Spring Viremia of Carp (SVC) virus

Specimen (scope and application)

Potentially virus infected monolayer cell cultures (FHM, EPC, and BF-2)

Reagent

Lyophilised SVCspecific monoclonal antibody:

Preparation:

- Dissolve the lyophilised virus-specific monoclonal antibody in 500 µl of distilled water. Aliquot into portions of 50 µl and freeze in glass containers at –20 ºC.
- Dilute the stock solution 20-fold with 1x washing solution.

Procedure

1. Remove the culture medium by turning the plate upside down into disinfectant.
2. Use auto-pipette with tips to gently transfer 500 µl of fixative per well.
3. Incubate the plate at 4 ºC for 20 minutes.
4. Remove the fixative by turning the plate upside down and placing it face down on absorbent paper for a few seconds.
5. Pipet 1 ml of diluted washing solution into each well and wait for about 3 minutes.
6. Repeat step (5).
7. Add 200 µl of diluted monoclonal antibody solution to each well.
8. Incubate at room temperature for an hour.
9. Repeat steps (5)-(6).
10. Add 200 µl of diluted conjugate to each well.
11. Incubate at room temperature for an hour.
12. Repeat steps (5)-(6).
13. Quickly deposit 500 µl of filtered chromogen mixture into each well.
14. Incubate for 30-60 minutes at room temperature in darkness.
15. Examine the cell layer with an inverted microscope.

Electronmicroscopy

This is required for the morphological identification of viruses isolated from cell cultures or where viral inclusion bodies have been observed in histological sections. There are several techniques in electronmicroscopy (EM): ultrathin section transmission electronmicroscopy (TEM), negative staining EM and scanning electronmicroscopy (SEM). TEM and negative staining is useful for fish virology work. Specialised techniques for preparing the tissue sample for EM are required. Fixation of fresh tissue in glutaraldehyde or formalin fixed paraffin embedded histology blocks may be used to harvest the 'lesion' of interest. Glutaraldehyde fixed material provides the best virus preservation for morphological studies while paraffin embedded harvests are adequate. TEM is used to confirm that a virus is present in a lesion or inclusion body. PCR only indicates if the DNA is present in the sample, and *in-situ* hybridisation (ISH) is required together with PCR to show involvement in a lesion if TEM is not used.

Molecular Methods

In recent years, the advancement of genetic testing or molecular biology has provided a very useful tool in the identification of disease pathogens including viruses. A discussion of the methods is found in the section following.

Molecular Diagnostics

Molecular diagnostic methods employ the detection of fragments of genetic material (DNA or RNA) specific to an organism with or without amplification of very small copies of target genetic material in the original sample. It includes the polymerase chain reaction (PCR) for DNA, reverse transcriptase polymerase chain reaction (RT-PCR), nested PCR and real-time PCR which amplify the target gene fragment or primer sequence. Hybridisation tests include in-situ hybridisation (ISH) such as digoxigenin (DIG)-labelled DNA probes, and Dot-Blot hybridisation – which do not amplify the sample target gene.

Its usefulness includes it being applicable to all pathogens with known specific gene sequences – bacteria, virus, fungal and parasites. PCR tests are highly sensitive, being able to detect minute amounts of target pathogens through its amplification process. It is also highly specific where appropriate primer sequences, positive and negative controls, and optimisation of thermocycling conditions are used. PCR tests have produced very rapid results - in hours rather than days when compared with conventional culture methods.

Limitations of molecular tests are:

- Inability to detect agents new or emerging until appropriate primer sequences develop.
- Risk of false positives due to sample contamination. A positive result requires careful interpretation. Apart from sample contamination, it could mean the detection of non-viable virus genes on the sample, that is, it does not necessarily mean that there is an active disease process, unless other pathological, immunological and cultural techniques confirm the presence of live virus activity.
- Expensive: it requires a high level of technical skill and dedicated equipment, reagents and laboratory environment to operate optimally.

Sample requirements are fresh, frozen or 80-90% ethanol preserved tissue samples. Histology samples are useful for ISH, perhaps for PCR if DNA is not degraded.

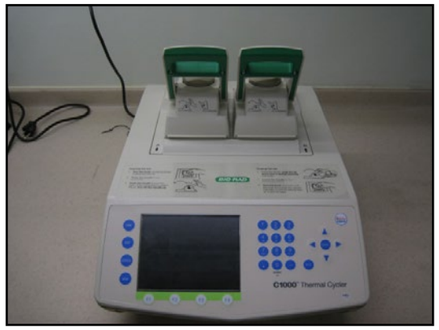


Figure 102. PCR thermocycler.

Aspects of performing a PCR test using a thermocycler (Fig. 102) include:

- Development or obtaining specific primers or target DNA sequences for the pathogen of interest.
- Obtaining positive control material for the pathogen of interest.
- Development of thermocycling conditions to optimise the annealing temperatures and times so as to obtain consistent and high yielding amplification of the target DNA product. This will vary with the laboratory equipment and reagents used.
- Preparation of the master mix of polymerase enzymes, nucleotides, and primers.
- Running the thermocycler which provides high temperatures (40-70 °C) for annealing and cooling cycles, and conditions for amplifying the DNA target product.
- Separating out the DNA target product through the gel electrophoresis technique. Visualisation of the gel for banding of the DNA target product (Fig. 103).
- A presumptive positive result if there is identification of the target DNA product as having the same molecular size in base pairs to the positive control, i.e. the same position in the gel.
- Sequencing of the DNA target product and comparing it to the published database (BLAST) is required to confirm the character of the product as belonging to the target pathogen.

- Validation – this involves comparing results of PCR runs with other test methods, e.g., immunology, cell culture and histopathology so as to determine the sensitivity and specificity limits of the test. The results of validation enable further selection and development of more useful primers for the pathogen of interest.

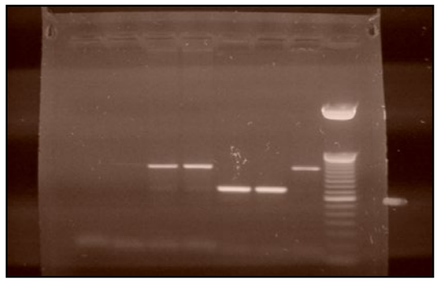


Figure 103. Gel electrophoresis for PCR testing.

The *In-Situ* Hybridisation (ISH) test is a very useful method of localising target pathogens in tissue sections. This is very complementary to a gel-PCR or real-time PCR whereby any doubt about the validity of a positive PCR can be clarified. A PCR may be a true positive if the ISH is also positive for the target pathogen.

The ISH requires the histological section of interest, specific DIG-labelled DNA probes of the target pathogen and a chromogen for detection of positive labelling of the probe with the target DNA in the tissue section. As there is no amplification of target DNA in ISH, the sensitivity of the test is lower than that for PCR tests.

Potential issues with ISH are:

- Background staining or exogenous staining which may make it difficult to interpret definitive probe and target DNA reactions.
- Lack of sensitivity to lower level infections producing false negatives.
- Degradation of the target DNA in tissue sections due to inadequate fixation or excessive exposure to acid and other decalcifying solutions.
- The specificity of the probe is critical to this test. A poorly defined probe may produce erroneous results.

Immunodiagnostics

Immunodiagnostics include the following tests:

- Immunohistochemistry test (IHCT)
- Indirect immunofluorescent antibody test (IFAT)
- Enzyme linked immunosorbent assay (ELISA)

Fish serology is a relatively new field and fish are different from mammalian species in that fish have the IgM class of antibodies and the responses to antigenic stimulation is variable depending on the species of fish and environmental temperatures. This affects the timing and consistency of antibody responses, making it difficult to develop reliable tests. Nevertheless there has been development of very sensitive immunodiagnostics for the detection of viral disease.

Immunohistochemistry Test (IHCT)

The IHCT is an antigen capture test targetting pathogens located in tissue. Specific antibodies to the pathogen are raised by inoculating rabbit or mouse hosts with the fish pathogen. The presence of a target antigen enables binding of the antibody and this allows a chromogen reaction to take place, e.g., immunoperoxidase using horseradish peroxidase. The result is colourisation of the target antigen on the cell surfaces, indicating a positive result. The key advantage of this test is the ability to visualise the distribution and relative intensity of infection with the target pathogen.

Potential issues with IHCT are:

- Cross reactivity with non-target antigens producing false positive results.
- Lack of sensitivity and failure to detect low-level infections or false negatives.
- The test requires very well preserved histological sections.
- The test section does not retain the positive reaction permanently and therefore cannot be archived for future reference. Photographic recording is required.

Indirect Immunofluorescent Antibody TTest (IFAT)

The IFAT is based on similar antibody-antigen reactions as in IHCT, except that the visualisation of this reaction requires the use of a fluorescent microscope, while the IHCT can be assessed using a standard light microscope.

Potential issues with the IFAT are:

- Non-specific background fluorescence due to tissue factors, e.g., ceroid, or other exogenous contamination.
- Specificity depends on the quality of the antibody, resulting in cross-reactivity problems.
- The test section does not retain the positive reaction permanently and therefore cannot be archived for future reference. Photographic recording is required.

Enzyme Linked Immunosorbent Assay (ELISA)

The ELISA is potentially a very useful test where as in the IHCT and IFAT tests, the specificity of antibodies for the target pathogen have been developed. The chromogenic reaction utilises an enzyme which is bound to the positive antigen-antibody complex either in sera or tissue sections. This is the main advantage over the IHCT and IFAT which require tissue sections. This means that non-lethal animal samples (blood and reproductive fluids) can be used.

Overall, immunodiagnostics take considerable effort to develop, particularly in the establishment and validation of specific antisera. They can be routinely employed and are very cost-effective for large-scale surveillance or health testing situations.

Clinical Pathology

The analysis of fish blood for changes in blood cell count and morphology (haematology) and detection of serum enzymes and electrolytes is the discipline of clinical pathology related to clinical physiology. The potential for the detection of disease states and abberations in fish physiology using haematology and serum biochemistry offers a non-lethal form of disease investigation. However, at present, there remain a number of obstacles to be resolved before this usefulness can be realised. These include:

- Establishment of reference values and their dynamics in different normal physiological states for the species of interest.
- Research into indicator enzymes for particular disease states.
- Research into assay methodologies to identify and resolve potential artifacts at sampling and testing.

The blood collection method is detailed in the necropsy procedure. It is important to note that artifacts can occur in the blood parameters due to:

- Inadequate or inappropriate use of anticoagulant.
- Haemolysis from forcing blood through the needle hub.
- Delayed assay if blood serum or plasma is not separated after collection.
- Anaesthetic effects.
- Delayed collection or collection from a dead fish.

Blood should be collected from an appropriately anaesthetised live fish, of a volume sufficient for the anticoagulant in the collection vial, spun down to separate the cell fraction from the plasma (for biochemistry) and refrigerated at 4 ^{0}C and assayed within 24 hours. Blood smears should be made as freshly as possible after collection, air dried and made ready for staining in Giemsa or Diff-Quik^{R}. Selected examples of blood cell types from cultured fish are presented in Figs. 104-118.

Haematology

Fish haematology parameters of clinical relevance are the packed cell volume (PCV), haemoglobin concentration (Hb), erythrocyte count and total leukocyte count. Due to the nucleated erythrocytes, automated cell counters cannot be used to calculate the fish erythrocyte cell counts. These values have to be manually counted or simple estimates derived from blood smears. Blood is routinely sent to a human clinical laboratory for assay of PCV and Hb values, while smears are examined in-house for morphological abnormalities in the erythrocytes, for the presence of blood-borne pathogens, e.g., haemoflagellates or marked changes in leukocyte proportions. Fish haematology is useful for the assessment of clinical anaemia and inflammatory responses. The application of fish haematology in the AFCD fish disease laboratory supplements the pathological and microbiological diagnostic work, but remains to be further developed and validated. An important principle to note is that the normal ranges of blood cells (i.e. morphology and numbers) can vary widely with species of fish and the physiological states of the fish. Therefore appropriate clinically normal fish are needed for comparison, especially where published reference ranges for parameters are lacking (Table 24).

Table 24. Reference values for fish haematology based on salmonids (adapted from Stoskopf (1993))

Hematocrit HT (%)	32-45
Haemoglobin Hb (g/dl)	7-9
Erythrocyte count (cells/ml)	1.2-1.7x10^{6}
Leucocyte count (cells/ml)	10-15 x10^{3}
Reticulocyte count (cells/ml)	1.8-2 x10^{3}
Thrombocyte count (cells/ml)	2.5-3 x10^{3}

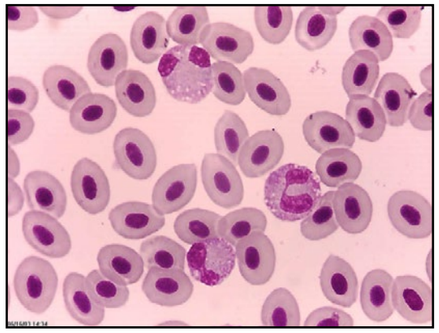


Figure 104. Red Cap Oranda Goldfish (*Carassius auratus*); Giemsa-stained blood smear showing three polymorphonuclear leukocytes (heterophil or neutrophil).

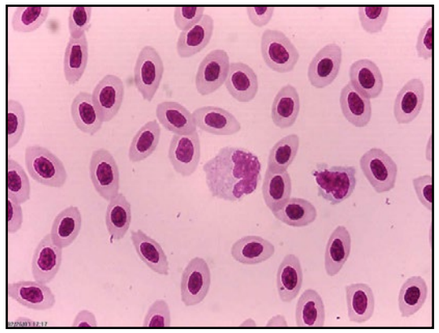


Figure 105. Brown spotted grouper (*Epinephelus areolatus*); Giemsa blood smear showing a polymorphonuclear leukocyte (heterophil or neutrophil). It has abundant cytoplasm and nuclear indentation. A mononuclear lymphocyte is adjacent with scant cytoplasm.

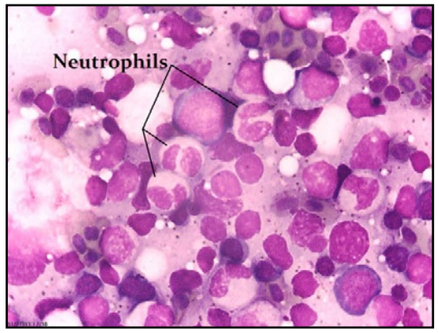


Figure 106. Neon tetra (*Paracheirodon innesi*); Giemsa-stained kidney smear showing a range of haematopoeitic cells including several polymorphonuclear leukocytes with segmented nuclei considered to be neutrophils or heterophils.

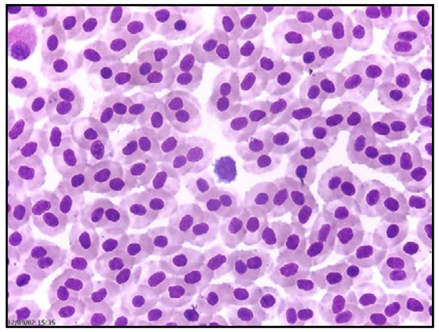


Figure 107. Yellow-finned seabream (*Sparus latus*); Giemsa-stained blood smear showing a mononuclear lymphocyte (centre) and a mononuclear eosinophil (top left corner).

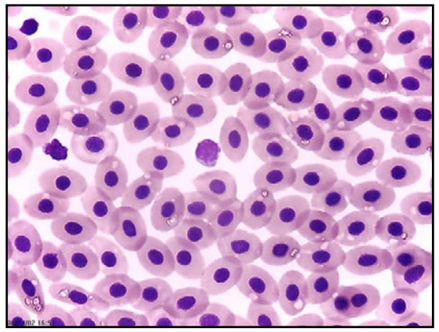


Figure 108. Koi carp (*Cyprinius carpio*); Giemsa-stained blood smear showing a mononuclear lymphocyte with a high nuclear to cytoplasmic ratio.

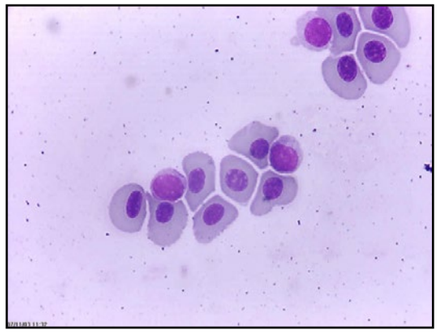


Figure 109. Goldfish (*Carassius auratus*); Giemsa-stained blood smear showing three mononuclear lymphocytes

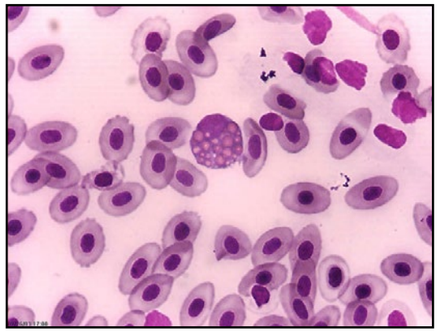


Figure 110. Discus (*Symphysodon discus*) gill blood; Giemsa-stained smear. Note the mononuclear eosinophil with prominent eosinophilic staining granules in the cytoplasm.

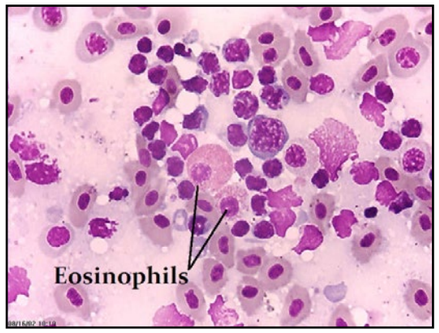


Figure 111. Red Seabream (*Pagrus major*) kidney; Giemsa-stained smear with two eosinophils.

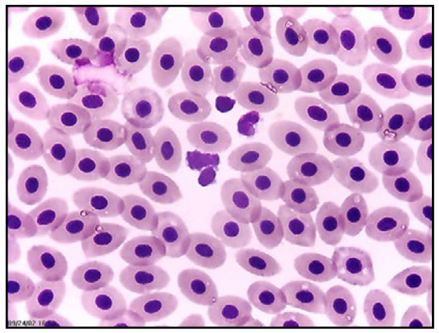


Figure 112. Koi carp (*Cyprinius carpio*); Giemsa-stained blood smear with thrombocytes.

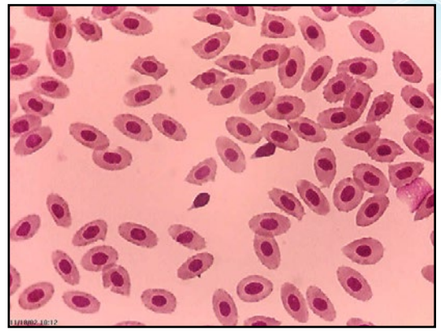


Figure 113. Yellow-finned seabream (*Sparus latus*); Giemsa-stained blood smear with two thrombocytes.

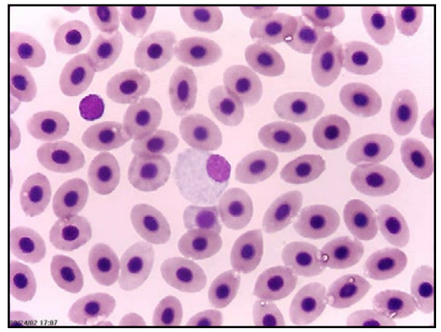


Figure 114. Koi carp (*Cyprinus carpio*); Giemsa-stained blood smear with a monocyte possessing a clear agranular cytoplasm (centre) and mononuclear lymphocyte to the left.

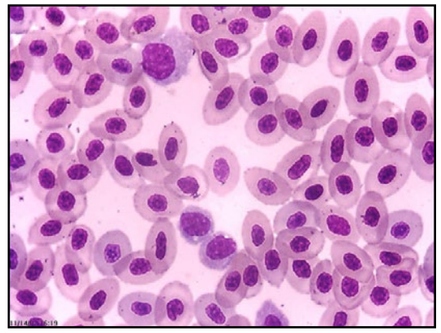


Figure 115. Giant grouper (*Epinephelus lanceolatus*); Giemsa-stained blood smear with larger mononuclear macrophage (top) and two smaller mononuclear monocytes (bottom).

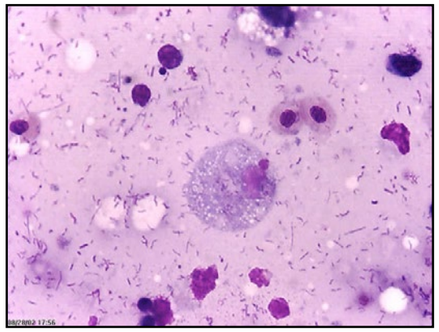


Figure 116. Mangrove snapper (*Lutjanus argimenticulatus*); Giemsa-stained skin lesion showing macrophage with phagocytosed bacteria and also plentiful free bacteria.

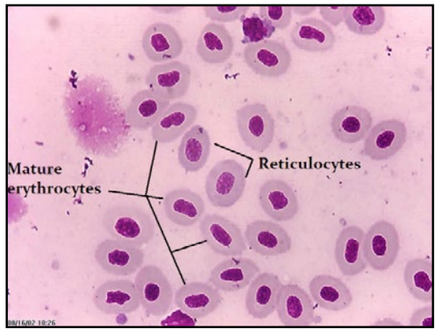


Figure 117. Snapper (*Lutjanus* sp.); Giemsa-stained blood smear showing reticulocytes which have a more rounded nucleus and cell shape with increased basophilia compared to mature erythrocytes with an ovoid nucleus and shape.

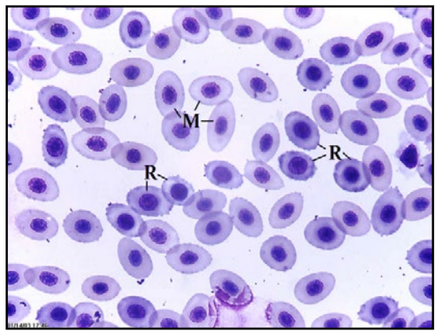


Figure 118. Green grouper (*Epinephelus coioides*); Giemsa-stained blood smear with reticulocytes(R) and mature erythrocytes (M).

The significance of haematological derangements must be supported by the clinical symptoms and pathology ensuring that one is aware of potential artifacts when interpreting results. The main indicators of derangements are:

Haematocrit (Packed cell volume): elevations – post surgery, acidosis, carbon dioxide exposure, dehydration in seawater, toxicosis (e.g., aromatic hydrocarbons, bis-Tri-N-butyltin, ozone, chlorine), male fish > female fish and seasonal variation; decreases – blood loss from infection, parasitism or trauma, kidney disease (nephrocalcinosis), poor body condition, clinical anaemia and its various causes, e.g., Vitamin E deficiency, rancid oils and fatty liver oxidation, toxicosis (e.g., nitrite, cadmium) (Stoskopf, 1993).

- Haemoglobin: elevations and decreases as for haematocrit.
- Erythrocyte count: elevations and decreases as for haematocrit.
- Reticulocyte count: elevations – regenerative anaemia, seasonal erythropoeisis, kidney disease or infection/parasitism and acidosis
- Leucocyte count: elevations – infection (acute phase), toxicosis (e.g., benzo-(a)-pyrene); decreases – chronic infection, stress and toxicosis (e.g., ozone)
- Thrombocyte count: elevations – infection; decreases – increased clotting time caused by stress (cortisol administration) and toxicity (e.g., cadmium (Stoskopf, 1993).

Serum Biochemistry

Fish serum biochemistry is dependent on assay techniques which must take into account the lower temperature of teleost metabolism. In general, enzyme values obtained from assays at 37 ^{0}C are higher than those at lower temperatures and a correction factor is necessary. Enzyme and electrolyte values are subject to wide variations depending on the species and the particular physiological state when the sample is taken. All of these issues make interpretation of results problematic. To assist in validating results, it is useful to assay control fish, i.e., fish not diseased and from similar cohorts or batches. Reference values for some fish species are presented in Tables 25-28. The AFCD fish diseases laboratory routinely collects blood for analysis by an external medical clinical pathology laboratory.

The significance of serum enzyme, metabolite, electrolyte and hormonal changes in fish must be correlated with the histological and pathological derangements while keeping in mind sampling artifacts. The main indicators of important derangements are:

- ALP (alkaline phosphatase): elevations – spawning, feeding and increased metabolic acitvity e.g., osmoregulation involving liver, kidney and intestines; decreases – infection.
- Total protein: elevations – acute infection, dehydration and toxicosis (e.g., chlorine) (Stoskopf, 1993); decreases – malnutrition, protein loss, chronic infection, liver or kidney injury and inappetance
- Albumin: elevations – dehydration; decreases – infection and liver injury.
- ALT (alanine aminotransaminase): elevations – liver injury, starvation, infection and toxicosis (e.g., carbon tetrachloride, formalin, potassium permanganate and microcystin (Powell, 2006).
- AST (aspartate aminotransaminase): elevations – nephrocalcinosis
- Glucose: elevations – stress and carbohydrate overload in feed; decreases – infection and toxicosis (e.g., aromatic hydrocarbons) (Stoskopf, 1993).
- BUN (blood urea nitrogen): elevations – gill or liver injury; decreases – starvation and liver injury.
- Triglygerides: elevations – high fat diet; decreases – nephrocalcinosis and starvation.
- Bilirubin: elevations – haemolysis/jaundice, anaemia and infection.
- Cortisol: elevations – stress, e.g., handling, crowding, surgery, etc.; decreases – infection.
- Calcium: elevations – toxicosis (e.g., chlorine) and saltwater exposure; decrease – acidosis.
- Sodium: elevations – osmoregulatory imbalance, saltwater exposure, toxicosis and acidosis; decrease – freshwater exposure, infection, toxicosis (e.g., chlorine and ozone) (Stoskopf, 1993) and softwater.
- Potassium: elevations – infection, saltwater exposure and toxicosis (e.g., nitrite) (Stoskopf, 1993); decreases – infection, freshwater exposure and stress.
- Chloride: elevations – saltwater exposure and chlorine toxicosis; decrease – toxicosis (e.g., ozone, dehydroabietic acid and aromatic hydrocarbons) (Stoskopf, 1993), infection and soft water.
- Globulin: elevations – infections (acute); decreases – post-infection and immunodeficiency.
- Creatine kinase: elevations – myopathy, physical injury and sampling artifacts.

Table 25. Reference values for fish serum biochemistry based on salmonids (adapted from Cameron (1992), Stoskopf (1993) and Ferguson (2006))

Serum / blood parameter	Reference values
Alkaline Phosphatase ALP (U/L)	600-1200
Total protein (g/L)	28-60
Albumin (g/dl)	4-7.5
Alanine Aminotransferase ALT (U/L)	7-12
Aspartate Aminotransferase AST (U/L)	270-340
Glucose (mg/dl)	63-144
Blood Urea Nitrogen BUN (mg/dl)	3.5-4.1
Triglycerides (mg/dl)	600
Bilirubin, Total (mg/dl)	0-2
Cortisol (μg/dl)	1.5-18.5
Calcium (mmol/L)	11.1-12.8
Sodium (mmol/L)	123-164
Potassium (mmol/L)	3.2-3.5
Chloride (mmol/L)	120-147
Globulin (g/L)	11-41
Creatinine Kinase CK (U/L)	549-3425

Table 26. Reference respiratory and acid-base values for resting, non-diseased fish under normoxic conditions. Values for freshwater (fw) and saltwater (sw) fish (Ferguson, 2006).

Species	P_aO_2 (mmHg)	P_aCO_2 (mmHg)	pH	Hct (%)	Hb (g/100 ml)
Rainbow trout (*Oncorhynchus mykiss*)(fw)	133.2	2.0	7.85	22.5	6.74
Atlantic salmon (*Salmo salar*)(sw)	110.0	1.95	7.90	26.0	10.0
Common carp (*Cyprinus carpio*) (fw)	23.2-38.0	2.43	7.92	33.6	10.37
Yellowfin tuna (*Thunnus albacares*)(sw)	79.2	3.90	7.85	33.4	-
Starry flounder (*Platicchthys stellatus*)(sw)	34.9	2.0-3.0	7.95	22.5	-
European eel (*Anguilla anguilla*) (fw)	49.1	3.0	7.92	-	17.2

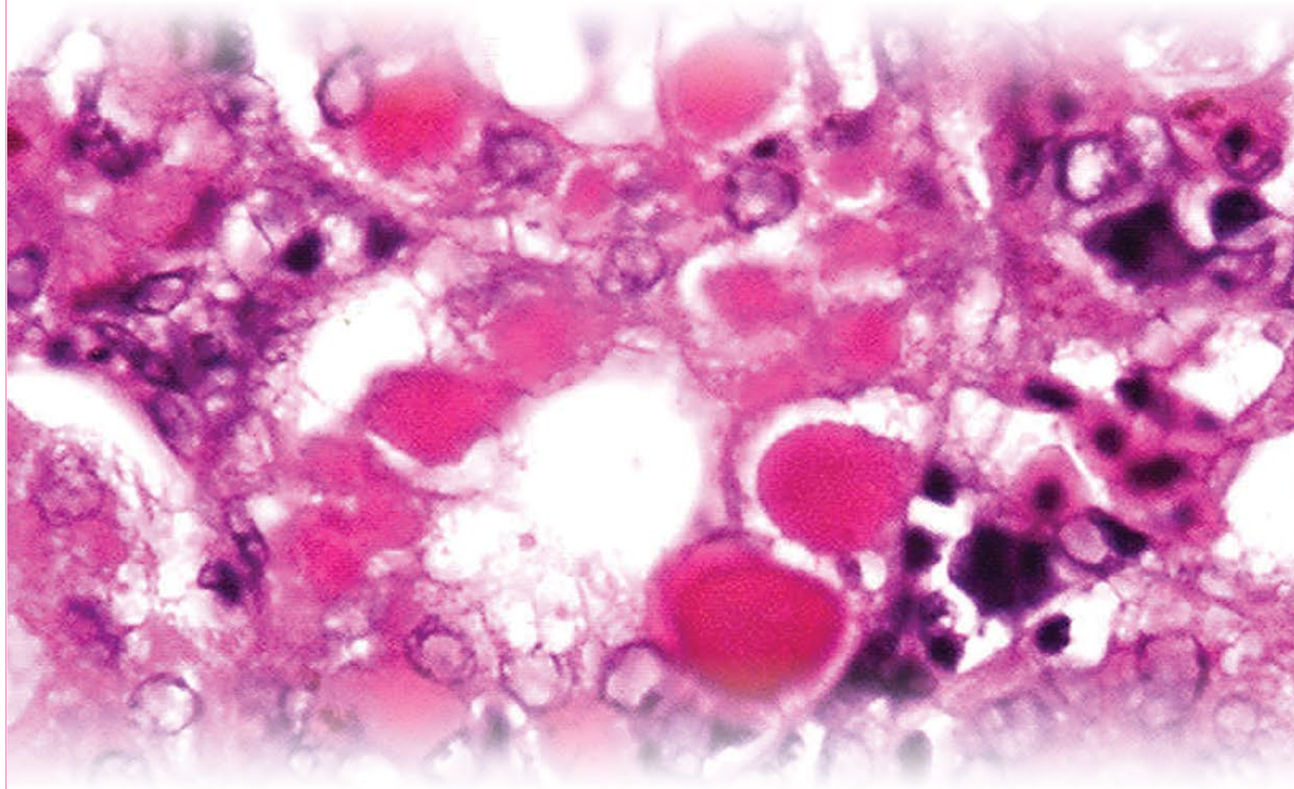
Table 27. Reference values for plasma electrolyte, glucose, lactate and osmolality values for freshwater (fw) and saltwater (sw) fish (Ferguson, 2006)

Species	Na^+ mM	K^+ mM	Ca^{2+} mM	Cl^- mM	Glucose mg/dL	Lactate mg/dL (mM)	Osmo-lality mmol/kg
Rainbow trout (*Oncorhynchus mykiss*) (fw)	123-164	3.3-3.5	5.5-6.4	120-147	63-144	4-17	296-340
Atlantic salmon (*Salmo salar*) (sw)	140-165	-	3.5-12.5	-	-	(2-4)	-
Brown trout (*Salmo trutta*) (fw)	-	-	-	109	52	-	-
Brook trout (*Salvelinus fontinalis*) (fw)	157	2.5	3.0	127	-	(3.1)	321
Lake trout (*Salvelinus namaycush*) (fw)	157-174	1.0-6.0	2.7-3.5	-	76-100	-	-
European eel *(Anguilla anguilla)* (fw)	143-150	1.8-2.3	2.3	88	-	-	-
European eel *(Anguilla anguilla)* (sw)	164-183	3.2-3.4	2.4-5.5	140	-	-	-
American eel *(Anguilla rostrata)* (fw)	138-151	2.8	3.2-3.3	70-111	-	-	252-308
Northern pike *(Esox lucius)* (fw)	122-128	2.0-2.2	5.0-5.6	105	25-71	-	274
Channel catfish *(Ictaluris punctatus)* (fw)	139	3.6	3.4	114-131	29.1-64.5	-	-
Koi carp *(Cyprinus carpio*) (fw)	142	2.0	6.0	107	111	-	293
Flounder *(Paralichthys spp.)* (sw)	180	4.0	3	160	-	-	337
Smallmouth bass *(Micropterus dolomieu)* (fw)	128-140	8-10	5-7	111-128	35-95	11.2-2.98	-
European river lamprey *(Lampetra fluviatilis)* (fw)	120	3.9	2.5	104	-	-	270
Sea lamprey *(Petromyzon marinus)* (sw)	156	32	3.5	159	-	-	333

Table 28. Reference values for liver function serum chemistry (Ferguson, 2006)

Species	ALT (U/L)	AST (U/L)	ALP (m/L)	LDH (mmol/L)	Creatinine mg/dL	T. Biliru-bin mg/dL	Total protein g/dL
Rainbow trout (*Oncorhynchus mykiss*)	7-12	158-368	50-200	250-100	0.2-0.5	0-2	-
Goldfish (*Carassius auratus*)	106	900	-	-	-	-	3.4
Koi carp (*Cyprinus carpio*)	-	-	-	-	0.56	-	4.2
Channel catfish (*Ictalurus punctatus*)	17.5	95	20	172	-	0.4	2.2-4.0
French grunt (*Haemulon flavolineatum*)	2.5	30	-	-	0.2	0	3.2
Blue tang (*Paracanthurus hepatus*)	5.5	19.2	-	-	1.9	0.08	29.
Dover sole (*Solea solea*)	<29	<89	-	-	-	0-0.14	5.7-6.2
Nurse shark (*Ginglymostoma cirratum*)	4.4	18.5	-	74	0.4	-	2.6-3.5

PART 3 – BACTERIAL DISEASES



PART 3 – BACTERIAL DISEASES

The discussion of diseases and pathology of fish will be presented in the following format, so as to enable the clinician to begin the investigation from the farm level right through to the laboratory work and reporting back to the farmer.

- Farm history
- Clinical signs
- Epidemiology
- Pathophysiology
- Diagnosis
- Control and Prevention

It is important to remember that the diagnosis of fish disease in a timely manner is dependent on these factors:

- An accurate history
- Appropriate samples
- Reliable laboratory tests
- Inpretation of all the clinical and diagnostic information in a logical and consistent manner based on a good understanding of the pathophysiology of the disease in question

Vibriosis

Farm History

Typically, farmers in the mariculture sector experience significant losses of fish in their grow-out net cages associated with vibriosis. Species groups infected by vibriosis include grouper, seabream, snapper and pompano. Vibriosis accounts for an estimated 68% of disease reported in grouper species (APEC/AAHRI/FHS-AFS/NACA, 2001).

Figure 119. Green grouper (*Epinephelus coioides*) fingerling mortalities due to mixed infection - vibriosis (*Vibrio alginolyticus*) with secondary flexibacteriosis (*Flavobacterium* sp.).

Clinical Signs

In a 6-month disease surveillance project during March to September 2000, of 418 marine cultured fish (consisting of 10 species) examined by necropsy, 57% were diseased and of these fish, 44% of the gross pathological changes were consistent with vibriosis – namely dermal ulceration, myositis, myonecrosis, exopthalmos, panopthalmitis, corneal damage, spleen enlargement, congestion and necrosis, anaemia (pale gills), heart haemorrhage, mouth-head ulceration, fin-skin erythema, and fin-tail necrosis (Austin and Austin, 1999).

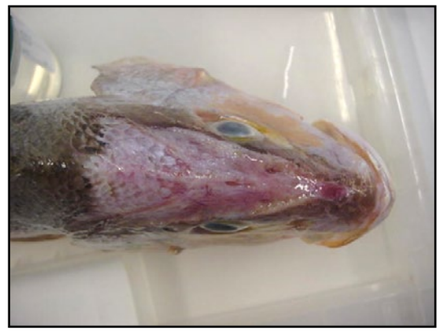


Figure 120. Snapper (*Lutjanus* sp.) with severe ulceration of the head and snout region.

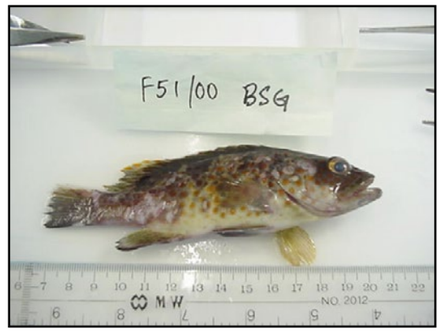


Figure 121. Brown spotted grouper (*Epinephelus areolatus*) grow-out sized fish with skin ulcerations due to *Vibrio parahaemolyticus* and *V. alginolyticus.*

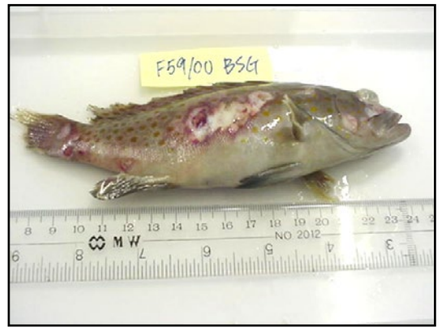


Figure 122. Brown spotted grouper (*Epinephelus areolatus*) near market sized fish with skin ulcerations, tail necrosis and exophthalmos, and panophthalmitis due to *Vibrio parahaemolyticus.*

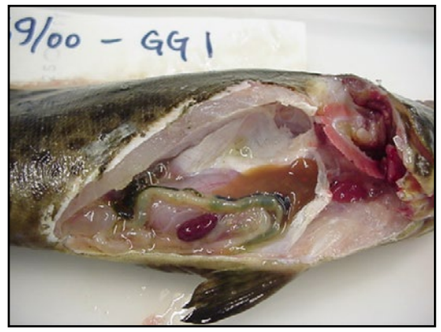


Figure 123. Green grouper (*Epinephelus coioides*) with splenic enlargement and congestion; *V. vulnificus* and *V. parahaemolyticus* were isolated from the spleen.

Figure 124. Green grouper (*Epinephelus coioides*) with pale gills and liver indicative of anaemia; *V. alginolyticus* isolated from kidney and spleen.

Figures 119-131 illustrate the various clinical signs (gross pathology) associated with usually mixed infection by one or more species of marine vibrio. These are subacute infections. In acute infections no external lesions are present, but infected fish become darkened and float listlessly in the water. Fish that die have gaping mouths, which is indicative of asphyxiation. Reported as “listless disease” by farmers, the fish die with mouth and gill covers open wide and the raphe of the lower jaw may be split apart. Also the fish become darker, and unresponsive to external stimuli. Whether this condition is due solely to vibrios is debatable as other bacteria are also isolated from internal organs.

Figure 125. Green grouper (*Epinephelus coioides*) fingerling with body darkening, no external ulcerative lesions and death with signs of asphyxiation; *Vibrio parahaemolyticus* and *V. fluvialis* were isolated from kidney and spleen respectively. However, *Photobacterium damsela* and *Pseudomonas* sp. were also isolated.

Figure 126. Red seabream (*Pagrus major*) with haemorrhagic dermal ulceration, corneal necrosis and perforation; *V. alginolyticus* was isolated from the skin lesion and spleen.

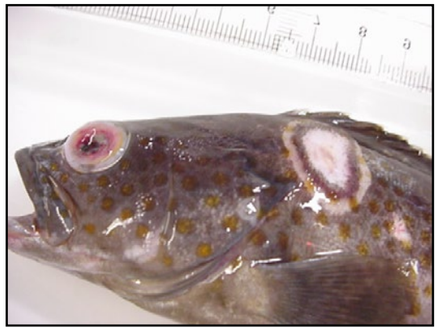


Figure 127. Brown spotted grouper (*Epinephelus areolatus*) with corneal ulceration and a healed circular skin lesion; *V. alginolyticus* was isolated from eye, kidney and spleen.

Figure 128. Giant grouper (*Epinephelus lanceolatus*) with extensive ulcerative myonecrosis; *V. alginolyticus* was isolated from the kidney and skin lesion.

Figure 129. Mangrove snapper (*Lutjanus argentimaculatus*) with myonecrosis; *V. alginolyticus* was isolated from the skin lesion, kidney and spleen.

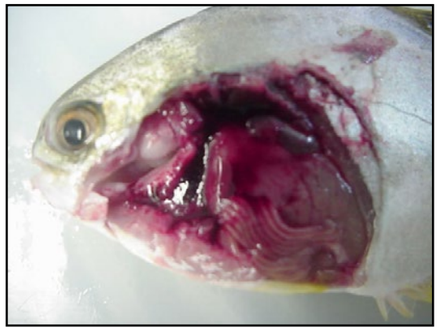


Figure 130. Pompano (*Trachinotus blochii*) with kidney haemorrhage; *V. alginolyticus* was isolated from the kidney.

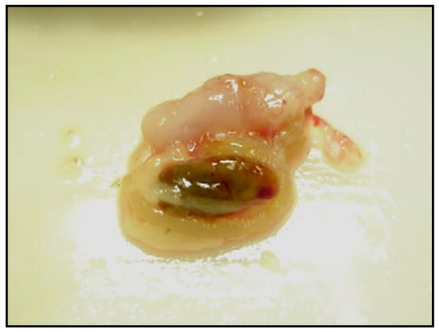


Figure 131. Brown spotted grouper (*Epinephelus coioides*) spleen showing necrosis and brown discolouration; *V. alginolyticus* was isolated from the spleen.

The clinical signs described are not pathognomonic for vibriosis but represent invasion of the external integument by an opportunistic pathogen ubiquitous in the marine environment.

Epidemiology

Typically, vibriosis outbreaks occur in recently translocated fish (Figs. 132-134). These would be fingerlings purchased by the farmer traditionally in late spring to early summer to coincide with rising water temperatures, the rationale being that fish growth rates are optimised as fish eat more at warmer temperatures. Vibriosis occurs in association with periods of fluctuations in salinity, increased organic load or stress brought on by net changing and grading of fish, with the period following initial stocking being critical (Leong and Colorni, 2002).

Vibriosis is reportedly a summer disease or more severe at high water temperatures (Stoskopf, 1993; Actis *et al*., 1999; Roberts, 2001; Noga, 2010). The literature provides some evidence that concentrations of vibrios are influenced by water temperature, being higher at elevated temperatures and lower or absent at lower or cold temperatures in coastal waters (Wright *et al*., 1996; Barbieri *et al*., 1999; Bertilsson *et al*., 2006). For example, *Vibrio* spp. concentrations (< 1 – 1000 CFU/ml) in estuarine waters was positively correlated with a water temperature range of 0.9-30.7 ºC (48% of variability, [R2] = 0.4819) and negatively correlated with dissolved oxygen levels. Together with water temperature, estuarine bacteria, phosphorus, ammonia nitrogen, salinity, turbidity and pH levels account for 67% of the variability associated with the isolation of vibrios from the environment (Pfeffer *et al*., 2003).

From local data, dissolved oxygen within mariculture farms in March to August (spring to summer) has a mean of 6.5 mg/L (n=56, range 2.2-10.1 mg/L), which is statistically lower (p=0.045, t-test) than in September to February (autumn to winter) with a mean of 7.5 mg/L (n=24, range 4.8-12.1 mg/L). However both mean values are adequate to maintain fish health, but there may be occasions in some mariculture zones when DO is suboptimal (< 5 mg/L).

From local data, seawater from within mariculture farms produces water presumptive vibrio counts from March to August (spring to summer) of a mean 4,131 Colony Forming Units (CFU)/ml (n=131), a range of 20-180,000 CFU/ml and from September to February (autumn to winter) of a mean 747 CFU/ml (n=50), and a range of 0-10,000 CFU/ml with p = 0.28, t-test; which is not statistically different. When seawater from outside the limits of the farms were analysed, presumptive vibrio counts from March to August (spring to summer) of a mean 566 CFU/ml (n=122), a range of 0-11,000 CFU/ml and from September to February (autumn to winter) a mean of 152 CFU/ml (n=48), and range of 0-1,400 CFU/ml with p = 0.04, t-test, were recorded, which is consistent with the literature that higher vibrio concentrations occur in the warmer months of the year. Collectively these data sets suggest that for fish farms, factors other than seasonal variation affect vibrio concentrations. These data also indicate that marine vibrios are present throughout the year in Hong Kong. Therefore disease outbreaks due to vibrios can occur whenever fish held in these waters are stressed.

The transportation of young fish can produce stress. Major stress factors in packing water quality include low DO, elevated ammonia, carbon dioxide and either high (>28 ºC) or low (<18 ºC) water temperature and poor handling. High ammonia in the water results in increased blood ammonia and toxicity. Elevated carbon dioxide can interfere with the ability of fish blood to carry oxygen (FAO, 1988). Fish can be packed at a high density of about 100 fish per bag with limited water for at least 10-12 hours and transport delays can occur. This can result in degradation of the water quality. For example, the vibrio counts can be high, with a mean of 14,670 CFU/ml, n=3, range 1,350-40,400 CFU/ml (optimum < 200-400 CFU/ml), and in one example, the carbon dioxide reached 59.2 mg/L (optimum < 15 mg/L) and unionised ammonia was 1.94 mg/L (optimum < 0.035 mg/L).

Figure 132. Unloading grouper fingerlings, which were airfreighted from Taiwan; note the cloudy weather, which is ideal for handling fish.

Figure 133. Bags of fish are floated in a sea-cage to acclimatise fish to temperature differences, which reduces stress on fish.

Figure 134. Counting and sorting of fingerlings; placement in floating baskets prevents the fish from diving to the bottom of the sea-cage immediately and forming a clump, which is another way to reduce stress on fish.

Mortalities due to vibriosis can begin within a week of stocking, particularly if the unloading process is stressful to the fish, e.g., during the heat of the day, and if fish are stocked in crowded cage pens. A 6-month survey was conducted in 2000 of four farms from four locations at Yim Tin Tsai (West), Tai Tau Chau, Kat O and Low Tik Wan, in which normal and sick fish over the period of the survey were sampled for laboratory examination and bacterial culture. The reported average cumulative mortality rates in stocked fish up to 14 weeks post-stocking were averaged at 58% (range 35 – 85%). Reported grouper fish species mortalities due to vibriosis averaged 85% (range 58-93%) while non-grouper species mortalities were 51% (range 29-68%). These figures are estimates only as other bacteria are sometimes involved in vibrios infections.

The “listless disease” is an acute disease, characterised by body darkening, inappetence and rapid death with no obvious body lesions. It can kill greater than 80% of fingerlings in a week. Farmers cannot treat this condition using medicated baths as they do for ulcerative vibriosis. From the time symptoms first appear, it takes 5-6 hours for fish death to occur. Farmers report anecdotally that when the fish are placed near floats with seaweed and barnacles, the reported mortality is less, i.e. 30-40% survival compared to doing nothing when only 10-20% survive in an outbreak. One possible hypothesis is that there is an improved ‘microclimate’ for the fish, which may be associated with filter feeders removing organic matter from the water. The Fisheries Branch of AFCD conducted a study on installing artificial reefs to act as biofilters which provide a hard stratum for growing filter feeders such as green-lipped mussels to trap and remove organic wastes, thereby improving the water quality. The study findings showed that the biofilters have led to the establishment of a rich fish community and helped remove organic wastes as well as improving the seabed environment within the fish culture zone. Farmers report that larger fish have better survival rates during vibriosis compared to smaller fish. Some farmers practise stocking in late winter and autumn with reduced severity of vibriosis and improved survival rates.

Pathophysiology

Infection by vibrios can take place in the following ways:

- Skin and eye abrasions from rough handling, fish to fish aggression, and overcrowding of fish
- Gill damage allowing colonisation and entry of bacteria

Once the integumentary barrier is breached, vibrios track through the connective tissue planes, releasing toxins, which cause necrosis of host tissues. This is most evident in ulcerative dermopathy, myonecrosis (Fig. 135) and corneal ulcerations. Fish mortality is the result of a combination of toxaemia and osmoregulatory failure from exposure of soft tissues to the environment. Should treatment with antimicrobials arrest the spread of the ulcerations, the wound can heal up with a covering of fibrous connective scar tissue, which is white in colour and scaleless (Fig. 127). Systemic vibriosis can result in splenic and kidney haematopoietic necrosis with haemorrhage (Figs. 136-138) and resultant anaemia.

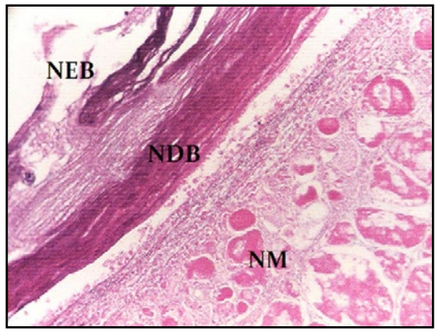


Figure 135. (H&E) Brown spotted grouper (*Epinephelus areolatus)* skin; necrotising epidermis with *V. parahaemolyticus* bacteria (NEB), necrotising dermis with bacteria (NDB) and necrotising muscle (NM) probably from bacterial toxin as fewer bacteria are observed but damage is extensive.

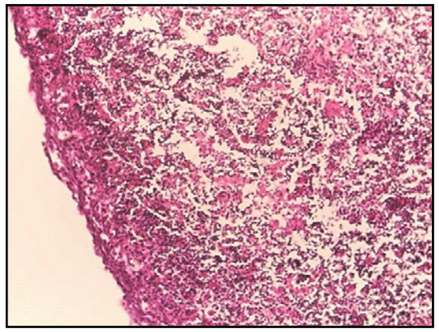


Figure 136. (H&E) Green grouper (*Epinephelus coioides*) with severe, generalised necrosis of the splenic pulp and haematopoietic tissues.

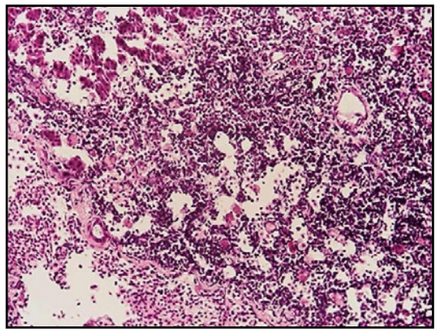


Figure 137. (H&E) Green grouper (*Epinephelus coioides*) kidney necrosis of the haematopoietic tissue; *V. alginolyticus* and *Vibrio* spp. were isolated from the kidney.

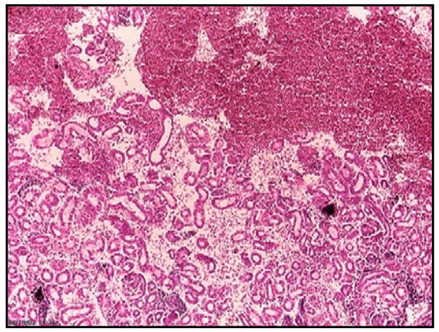


Figure 138. (H&E) Pompano (*Trachinotus blochii*) kidney showing haemorrhage; *V. alginolyticus* was isolated from the kidney.

With listless disease or acute vibriosis, potential toxins released from vibrios damage the erythrocytes in the gills. Damaged erythrocytes are observed to agglutinate in the lamellar capillaries resulting in capillary dilation. This is followed by phagocytosis of erythrocytes by macrophages in the dilated capillaries. Haemosiderin pigment from the breakdown of erythrocytes can be seen in the dilated capillaries, which contain effete erythrocytes, macrophages and haemosiderin (Figs. 139-140). It is plausible that blood flow in affected lamellar capillaries would be compromised, leading to gill perfusion deficits and is contributive to hypoxia. Damage to erythrocytes leads to anaemia in the fish, which also renders the fish susceptible to hypoxia. Similarly melanomacrophage centres (MMCs) or aggregates (Figs. 141, 144-145) are also seen in the head kidney and liver containing effete erythrocytes and haemosiderin. Damage to the heart also occurs in the form of pericarditis and myocarditis (Figs. 142, 143, 146a, 146b and 146c). Macrophage cells infiltrate and thicken the pericardium although this process occurs in the latter stages of the infection. The heart function is potentially compromised. Haemolysis is also observed in the heart blood with a large amount of ferritin-like crystals observed. Fish may die from hypoxia due to the combination of the haemolytic anaemia, disturbed gill perfusion and cardiac insufficiency. Deaths can therefore be sudden and fish may show no body lesions due to the rapid effect of the bacterial toxins.

Vibrios can produce extracellular toxins including haemolysins, proteases, haemagglutinins as well as a complex iron-uptake system (involving a siderophore called anguibactin). It is of advantage to the vibrios to haemolyse erythrocytes in order to obtain iron from the haemoglobin. This mechanism is reported in *V. anguillarum*. These virulence factors may also belong to warm water vibrios. Abscessation with liver necrosis, pericarditis, myonecrosis and macrophagic phagocytosis of gill bacteria can occur with *V. alginolyticus* isolated from tiger grouper (Figs. 146a,b).

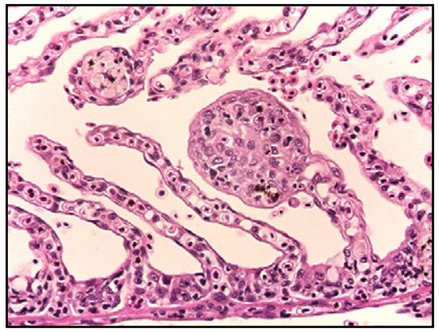


Figure 139. (H&E) Green grouper (*Epinephelus coioides*) gills with lamellar swellings containing macrophages, effete erythrocytes and haemosiderin deposits.

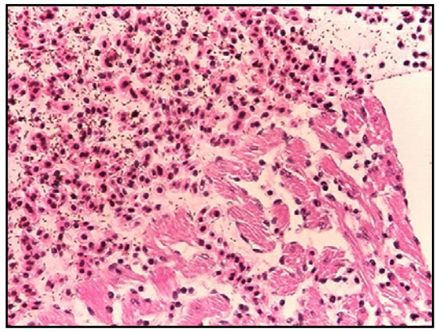


Figure 142. (H&E) Green grouper (*Epinephelus coioides*) heart; heart blood with ferritin-like crystals probably secondary to haemolysis.

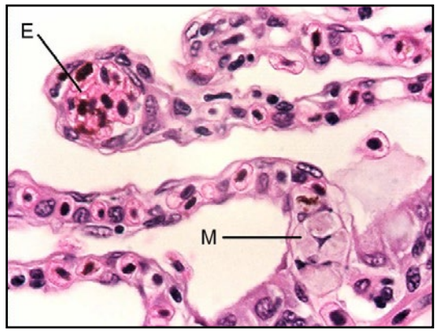


Figure 140. (H&E) Green grouper (*Epinephelus coioides*) gills with effete erythrocytes (E) agglutinated as a clump in the distal lamellar vessel and macrophages cleaning up erythrocytes in another lamellar vessel (M).

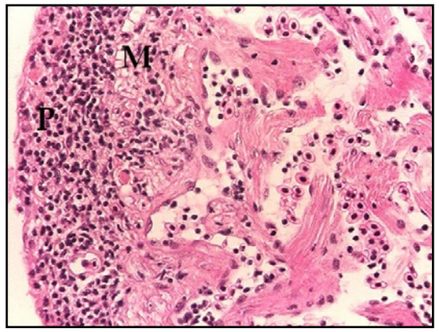


Figure 143. (H&E) Green grouper (*Epinephelus coioides*) heart with pericarditis (P) extending into a vacuolar myocarditis (M).

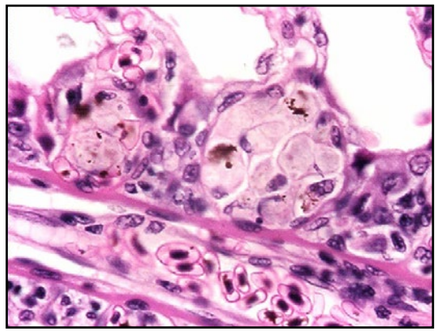


Figure 141. (H&E) Green grouper (*Epinephelus coioides*) gills with haemosiderin (dark brown pigment) in the melanomacrophages congregating in dilated blood capillaries of the basal lamellar area; melanomacrophages contain melanin, which can also be a dark brown-black colour.

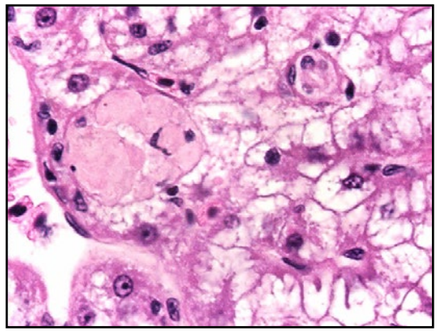


Figure 144. (H&E) Green grouper (*Epinephelus coioides*) liver - melanomacrophages aggregate similarly to those in the gills, indicative of a systemic haemolytic condition.

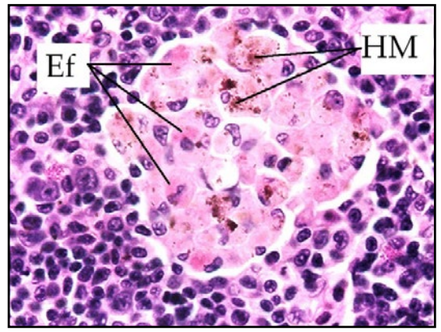


Figure 145. (H&E) Green grouper (*Epinephelus coioides*) liver with melanomacrophages aggregating with haemosiderin deposits (HM) and remnants of effete erythrocytes (Ef) staining slightly eosinophilic.

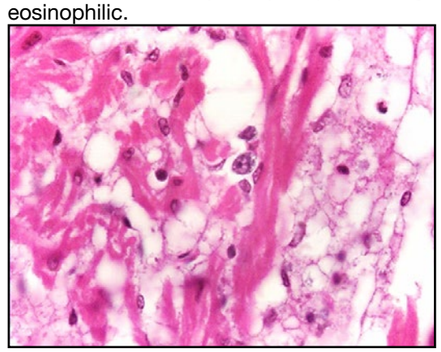


Figure 146a. (H&E) Green grouper (*Epinephelus coioides*) heart with myocardial degeneration, vacuolar changes and macrophages with phagocytosed material; *V. parahaemolyticus* was isolated from the kidney and spleen.

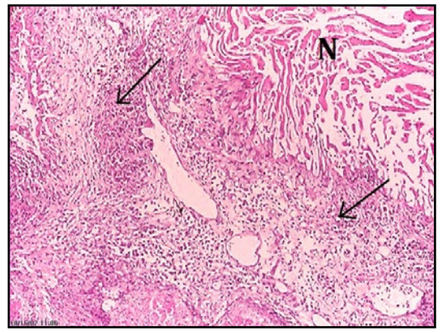


Figure 146b. (H&E) Tiger grouper (*Epinephelus fuscoguttatus*) with necrotizing myocarditis (arrows) due to *Vibrio alginolyticus* and normal myocardium (N).

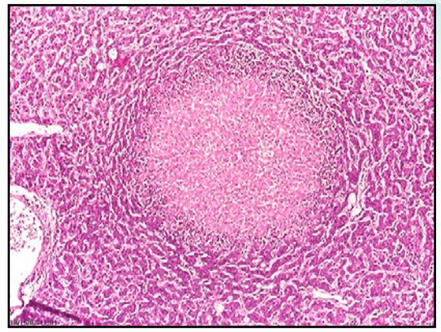


Figure 146c. (H&E) Tiger grouper (*Epinephelus fuscoguttatus*) with liver necrosis and abscesses due to infection by *Vibrio alginolyticus.*

Diagnosis

A difficulty often encountered in the diagnosis of vibriosis in marine fish is the fact that the bacteria are a part of the environment and normal flora of the gut. Simply isolating the bacteria or bacterium is not sufficient to support a diagnosis. The following considerations must be taken into account:

- Presence of internal and external pathology consistent with vibriosis
- Isolation of vibrios in the kidney and spleen (internal organs)
- Exclusion of other pathogens
- Identification of stress factors

It must also be noted that vibriosis is a secondary infectious disease and it is commonly encountered as a mixed infection with other pathogenic bacteria. It may not be possible to attribute the disease in the infected fish solely to vibrios.

Routine culture of kidney, spleen, skin lesions and eye lesions using Thiosulphate Citrate Bile Salt Sucrose (TCBS) Agar and marine agar is carried out for vibriosis. Incubation is for 2-3 days at room temperature (20-25 °C). Colonies may be green or yellow on TCBS and white on marine agar. Vibrios are Gram-negative, curved or straight short rods and motile. The vibriostat 0/129 is used to distinguish *Vibrio* spp. (which are inhibited by 0/129) from *Aeromonas* spp. The biochemical profiles for tropical *Vibrio* spp. are shown in Table 29.

Of the 171 bacterial isolates from 85 fish sampled for bacteriology in the 6-month survey of four mariculture farms in 2000, 103 isolates (60%) were vibrios. The *Vibrio* spp. isolated consisted of: *Vibrio alginolyticus* (37%), *V. parahaemolyticus* (23%), *V. vulnificus* (11%), *V. fluvialis* (9%), *V. cholerae* (5%), *V. mimicus* (1%) and untyped *Vibrio* spp. (15%). *V.*

damsela was also isolated in some disease cases. Of the 18 batches of fish (6 x *E. coioides*, 2 x *E. areolatus*, 1x *Trachinotus blochii*, 2 x *Rachycentron canadum*, 6 x *Lutjanus argentimaculatus* and 1 x *Pragus* sp.), 72% had vibriosis at some point during the 14-week post-stocking period. However, from 76 samplings, which included repeat samplings of 18 batches of fish monitored, 26% (20 samplings) were diagnosed with clinical vibriosis based on histopathology, gross pathology and bacteriology. Vibrios were frequently isolated from fish without clinical signs of vibriosis. This indicates that the mere presence of vibrios does not cause disease unless the fish are stressed by environmental factors.

Following species typing, antibiotic sensitivity testing (antibiograms) of the isolates is performed. The vibrios do show resistance to a number of antibiotics as shown in Table 30.

Table 29. Biochemical profiles of *Vibrio* species (adapted from Roberts (2001), Buller (2004) and Whitman (2004))

Vibrio species	Biochemical Tests						
	Vosges-Proskauer reaction	Utilize sucrose	Arginine dihydrolase	Utilize L-arabinose	Utilise D-sorbitol	Ornithine carboxylase	Indole
V.alginolyticus	+	+	-	-	-	+	+
V. parahaemolyticus	-	-	-	+/-	-	+	+
V.vulnificus	-	-	-	-	-	-	-
V.fluvialis	-	+	+	+	-	-	-
V.cholerae	+/-	+	-	-	+/-	+/-	+/-
V.mimicus	-	-	-	-	-	+	+

Table 30. Antibiogram profiles of Vibrios

Bacterium sp.	Sensitive	Resistant/Borderline
V. alginolyticus	amoxycillin-clavulanic acid, trimethoprim-sulphamethoxazole, doxycycline, erythromycin, neomycin, oxytetracycline, oxolinic acid, gentamicin, minocycline enrofloxacin, flumequine	doxycycline erythromycin, neomycin, oxytetracycline, oxolinic acid
V. parahaemolyticus	oxytetracycline, gentamicin, neomycin, erythromycin , amoxycillin-clavulanic acid, doxycycline, trimethoprim-sulphamethoxazole, flumequine, minocycline, erythromycin, enrofloxacin, oxolinic acid	erythromycin, neomycin
V. vulnificus	oxytetracycline, gentamicin, neomycin, erythromycin , amoxycillin-clavulanic acid, doxycycline, trimethoprim-sulphamethoxazole, flumequine, minocycline, erythromycin, enrofloxacin, oxolinic acid	erythromycin, neomycin
V. fluvialis	oxytetracycline, gentamicin, neomycin, erythromycin , amoxycillin-clavulanic acid, doxycycline, trimethoprim-sulphamethoxazole, flumequine, minocycline, erythromycin, oxolinic acid	neomycin
V. damsela	oxytetracycline, gentamicin, neomycin, amoxycillin-clavulanic acid, doxycycline, trimethoprim-sulphamethoxazole, flumequine, erythromycin, oxolinic acid	neomycin

Control and Prevention

Vibriosis outbreaks in fish occur because of stressors that favour the entry and establishment of pathogenic strains of vibrios. Therefore management of the disease must be centred on husbandry strategies that mitigate these stressors. Stressors for vibriosis include:

- Transportation and handling
- Temperature and salinity acclimatisation
- Stocking at high density
- Organic wastes in the environment
- High water temperatures
- Juvenile fish with naïve immunity

Transportation and handling

Usually farmers buy in fish by airfreight. These are normally packed at 95-100 fish per plastic bag with about 10 litres of water and oxygen filled, for 8-10 hours. On unpacking, although most fish are alive, up to 20% may be showing hypoxic stress – gaping mouth and operculae, surfacing and lethargic. To mitigate stress, the bags are placed in the seawater for 5 – 10 minutes to allow temperature acclimatisation. They are then debagged and placed into floating baskets at 180 per basket so as to prevent crowding behaviour by the fish as would happen if all the stock were placed in the net cage straight from the bags. Before this method of handling was introduced, many fish would die on the day of unloading due to hypoxia from their crowding behaviour. After 15 – 20 minutes in the baskets, the fish recover from the hypoxic stress and are swimming with more energy and look less dark.

The farmer suspends the raft net to give a 1.5m depth for better observation of the fish. If too much depth is given the fish will crowd at the bottom of the net cage and disease problems cannot be easily identified. The fingerlings are released into the net cage about 1 hour later when they have 'caught their breaths'. Feeding is withheld for 1-2 days to allow a rest period for the new stock and supplemental aeration is provided. All the rafts holding the fingerlings are covered in cloth sheets for sun-protection and to settle the fish.

Temperature and salinity acclimatisation

As there is no facility to temperature acclimatise transported fish upon arrival at farms, fish are discharged into the sea cages with a very short period of temperature adjustment or none at all in most farms. Temperature differences of a few degrees (up to 10 °C) between the fish bag water and the sea is a stressor, which affects fish survival and susceptibility to disease (Noga, 1996; Wedemeyer, 1996). The temperature acclimatisation required is a 1 °C change per hour or several hours for a 10°c difference (Noga, 1996; Wedemeyer, 1996). Fish often demonstrate stress by becoming a dark colour. Mortality rates of fish from Taiwan, Malaysia and Philippines averaged 70% while those from Mainland China (closer to Hong Kong) averaged 55%. This suggests that fish shipped over longer distances experience more transport stress and have higher mortalities, which. This is likely to be due in part to the failure of proper temperature acclimatisation and the build-up of metabolic wastes in the fish bags during prolonged transport periods.

Farmer reports suggest that differences in salinity between the source of the fish and that in the

culture zone can cause survival problems, and in the critical first few days, the fish have to adapt to temperature, oxygen, pH and salinity differences. During this period disease outbreaks are rapid (within a few hours) and severe (entire stocks have been lost despite treatment attempts with medicated baths).

To reduce such problems, prudent farmers try to organise fish deliveries to avoid periods of high temperatures (hot days) or when tidal flows are poor, but this is often beyond their control.

Stocking at high density

Crowding as a stressor can precipitate outbreaks of vibriosis (Langdon, 1988; Munday, 1988; Noga, 2010). High stocking density such as 3,000 fingerling fish per net cage of 3m x 3m x 3m leads to body abrasions and cohort aggression in species such as grouper. With more fish in a net cage, feeding, grading and treatment become less efficient. This results in more frequent grading to reduce size variations and bigger fish bullying smaller fish, as well as longer duration of handling with more fish to handle per cage. Feeding becomes less even in crowded cages with the potential for greater feed wastage. These stressors favour vibriosis. Lower stocking density of less than 1,000 fingerling fish per cage improves handling efficiency, reduces aggression, and leads to better feeding and less waste. This is better husbandry with less stress on the fish. For listless disease, management is conducted by reducing stocking density to about 500 fish per cage.

In the study of four farms the range of stocking density per sea cage (normally 27 m^3) was 500-3,000 fish at the start of stocking. The mortalities reported ranged from 17%-93% over a period of 14 weeks depending on fish species. Two farms with a stocking density of 1,500-3,000 reported mean mortalities of 84% while the other two farms with a stocking density of 500-1,500 reported mean mortalities of 58%. However, there was no statistical correlation between initial stocking density and mortality rates (r=0.48, p=0.057 ANOVA).

Assessment of fingerling stocking biomass and vibrio counts was performed to see if these had a causal relationship. The mean vibrio counts of water in the higher stocking density (2.5 kg/m3) fish culture zones (FCZs) was 571 CFU/ml (n=19), ranging from 80-1,700 CFU/ml while that in the lower stocking density (0.21 kg/m3) FCZs was 693 CFU/ml (n=28), ranging from 65-1,740 CFU/ml; these were not statistically different (p=0.46 t-test), nor correlated (r=0.16, p=0.27 ANOVA).

Based on this study, the initial fish stocking density in mariculture does not directly influence the risk of vibriosis and fish mortalities, but it would be worthwhile to extend the study to more farms, taking into consideration of other factors such as fish size at stocking and fish handling techniques. The study also indicated that the stocking density of fingerling fish measured in biomass/volume of cage is not correlated with vibrio concentrations. It would be useful to look at total biomass of fish in each culture zone and whether this affects the risk of vibriosis.

Organic wastes in the environment

Fish farming produces organic wastes discharged directly into the fish culture environment (Enger *et al.,* 1989; Wolfgang *et al.,* 2006). Vibrios feed and multiply on organic matter in the environment (Enger *et al.,* 1989; Vezzulli *et al.,* 2009) which may cause higher concentrations of pathogenic vibrios in the fish culture environment. There are on average higher concentrations of vibrios in the water of a fish farm zone than outside it (< 1 km). Farm vibrio counts average 4,131 CFU/ml; range: 0 – 180,000 CFU/ml, n=181 while vibrio counts outside farms average 449 CFU/ml; range: 0-11,000 CFU/ml, n=170, p=0.028, one-tailed t-test. Therefore the mean vibrio concentration in the water of marine farms is potentially nine times higher than background levels. The sources of vibrios are probably from the fish stocks and in the sediment of the fish farms. Culture of fish stomach and intestine with their contents produced mean presumptive vibrio counts of 710,075 CFU/g (n=63) with a range of 50-17,100,000 CFU/g. Algae from fish cages produced a mean presumptive vibrio count of 610,987 CFU/g (n=26) with a range of 1,500-7,700,00 CFU/g. Seabed sediment samples produced a mean presumptive vibrio count of 27,800 CFU/g (n=3) with a range of 5,400-40,000 CFU/g within fish farms and 6,225 CFU/g (n=4) and a range of 500-11,900 CFU/g outside fish farms (p=0.042, one-tailed t-test). Collectively these results indicated that vibrios are ubiquitous in the marine environment and higher concentrations occur in fish farms and fish can carry high concentrations in their gut.

Organic matter decomposition consumes oxygen in the aquatic environment, which may negatively impact fish health (Wolfgang *et al.,* 2006). To see if this is a significant risk in Hong Kong mariculture, a study of dissolved oxygen (DO) was conducted in 2000. In the study, mean dissolved oxygen levels in four mariculture zones were compared with that outside the farming zone (up to 1 km), which was used as the reference background. DO for farms

was 6.78 mg/L (n=80), ranging from 2.20-10.00 mg/L and this was not statistically different from that for outside farms which was 6.88 mg/L (n=64) with a range of 3.30-13.00 mg/L (p=0.37, one-tailed t-test). There was however variation between the mariculture zones studied. The mean DO in Kat O farms was 6.79 mg/L (n=8) with a range of 6.3-7.9 mg/L which was lower than the outside DO of 7.76 mg/L (n=7) with a range of 6.40-8.70 mg/L (p=0.046, one-tailed t-test). The mean DO in Lo Tik Wan farms was 5.89 mg/L (n=8) with a range of 4.4-7.3 mg/L, which was lower than the outside DO of 7.09 mg/L (n=8) with a range of 5.00-8.70 mg/L (p=0.046, one-tailed t-test). Nevertheless, the DO levels in Kat O and Lo Tik Wan are still adequate to maintain the health requirements for fish. These farms contrast with Yim Tin Tsai (West) and Tai Tau Chau where there was no significant difference in the mean DO in the farm relative to background levels. These results suggest that for some mariculture farms, there is a reduction in DO of about 1 mg/L and this may be related to the organic matter of the affected fish farms. However, dissolved oxygen is generally not lower inside marine fish farms relative to background estuary levels and thus is not likely a limiting factor for fish culture.

Reduction of organic wastes such as fish faeces and uneaten trash fish which are potential sources of vibrios entering the environment of the seabed and also regular cleaning of fish raft structures may reduce the concentrations of environmental vibrios and thus reduce the risk of vibriosis. Although DO is generally not a risk factor, it needs to be monitored by farms as it can decline to low levels, e.g., 2.2 mg/L. Farmers can achieve these in the following ways:

- Switching to more efficient feeds such as dry pellets that generate less wastage
- Regular cleaning of sea cage nets and rafts to remove biofouling algae
- Providing supplementary aeration to the cages during periods of low tidal flow or high water temperatures

High water temperatures

Fish are traditionally imported during late spring and summer when water temperatures may exceed 30 °C. Survey data suggest that outbreaks of vibriosis occur frequently in the warm summer months with rising water temperatures in the range of 25 – 30 °C. Thus fish mortalities from vibriosis are most common during the months of April to August. Bacteriology data indicate that vibrios grow best in warm conditions of 25 – 28°C. Warm temperatures may favour the pathogenicity of vibrios while causing increased stress to fish and triggering disease outbreaks.

Some farmers have switched to autumn to winter fish stocking and together with better handling of fish by spreading out the arrivals of fish over the year has been reported to reduce mortalities from disease in the 1st month of post-stocking.

Three field vaccination studies were conducted using green grouper (*Epinephelus coioides*) from 2001-2003. In the first study, fish were stocked in August 2001 and the recorded mean mortality of fingerlings in October 2001 was 48.9% (n=750 x 4 replicates) with a range of 29.7-56.4%. In the second study, fish were stocked in November 2001 and the recorded mean mortality of fingerlings in January 2002 was 15.6% (n=989 and 984) with a range of 14.8-16.3%. In the third study, fish were stocked in December 2002 and the recorded mean mortality of fingerlings in January 2003 was 24.7% (n=1031 and 1052) with a range of 18.6-30.8%. Statistically the autumn to winter stockings resulted in mean mortality of fingerlings within the first 2 months of stocking of 20.1% which was lower than for the summer stocking mortality of 48.9%, p=0.007, t-test. These results suggest a reduction in mortalities when fingerling fish are not stocked in summer when temperatures are high. Given that the prevalence of vibriosis is 72% from the AFCD study of four mariculture farms, it is likely that fish losses due to vibriosis is potentially minimised under Hong Kong conditions by a change in the season of stocking.

Juvenile fish with naïve immunity

Due to lower pricing, fingerlings of about 10 g (7.5-10 cm) are commonly imported. The immune system of fish become mature as the fish grows in size (Ellis, 1988). The younger and smaller the fish, the less developed the immune capacity (Iwama and Nakanishi, 1996) and the more susceptible they are to disease. Therefore importing and stocking small fingerlings with less ideal husbandry and environmental conditions increase the risk of loss due to disease. Some farmers have reported that stocking larger and older fingerlings improve survival rates, but this was also contingent on good husbandry. However the critical size at which grouper fingerlings are immunologically competent needs to be researched since field vaccination trial data using fish of 10-13 g did not reveal improved survival due to vaccination of the 13 g fish compared to the 10 g fish.

Antimicrobial treatment

Having managed the stressors through appropriate husbandry, a greater probability of a successful outcome in the antimicrobial treatment of vibriosis outbreaks in a cage of fish is possible. The following principles are key to antimicrobial treatment:

- Selection of the appropriate antimicrobial should be based on isolation of the vibrio(s) involved and based on the results of the antibiogram.
- Treatment should be commenced very early in the course of vibriosis. As the onset of body lesions progresses rapidly to mortalities, delays in treatment usually result in failure to control mortalities. This is contingent on the farmer observing his fish at least twice a day to look for the odd fish showing damage to fins, scales or eyes.
- While the fish are eating, antibiotics administered in the food is a better option than bath treatment as it is more effective in achieving therapeutic levels in the blood, less labour-intensive, less costly and can be maintained over the required period of treatment. There is also less potential residue impact on the seabed with in-feed medication compared to bathing because less drug is required for the former.
- Bath treatment is used when fish are not eating. This requires setting up a treatment canvass-bath, scooping fish from the net cage into the bath, setting up additional aerators and constant monitoring. The duration of treatment is limited to 30-60 minutes. Repeat treatments are required depending on the severity but usually are done every 2-3 days depending on labour availability and the number of cages to treat. The procedure can be stressful to the fish due to the handling. Usually the spent medicated bath is discarded into the sea and diluted away by the current.
- Withholding periods apply, although as the fish fingerlings will not be of market size for another 18 months to 2 years, antibiotic residues in treated are unlikely to be an issue.

Vaccination programme

Commercial vaccines have been trialled for the control of vibriosis in farms. The control of vibriosis through vaccination has been very successful in countries where cold water vibrio species are problematic.

Summary

Vibriosis is not an easy disease to manage because many stressor factors are potentially involved but at different levels. A combination of measures that reduce stress on the fish, improve the hygiene of the environment, immunisation, better fish handling and targeted medication are needed to reduce the impact of this major marine fish disease.

Streptococcosis

Farm History

Streptococcosis occurs in marine culture when infected fish come into contact with uninfected fish (Figs. 147-148). It also occurs in ornamental fish such as the Kuhli loach (*Acanthophthalmus kuhli*). In marine farms, it does not necessarily occur every year but is likely a culmination of stressors on the fish including co-parasitism (Fig. 159) in the infected batch of fish. Co-infections with *V. alginolyticus* and *Photobacterium damsela* are observed. In Hong Kong, greater amberjack (*Seriola dumerili*), giant grouper (*Epinephelus lanceolatus*), snapper (*Lutjanus* spp.), pompano (*Trachinotus blochii*) and cobia (*Rachycentron candaum*) are susceptible to streptococcosis.

The disease globally is caused by a variety of *Streptococcus* species including *Streptococcus iniae, Enteroccocus seriolicida* (Woo *et al.,* 2002), Lancefield group B or D *Streptococcus, S. faecalis and S. faecium* belonging to the Enterococcus group (Inglis *et al.,* 1993), and more recently *S. agalactiae* (Bowater *et al.,* 2012).

Figure 147. Greater amberjack (*Seriola dumerili*) mortalities on a marine farm.

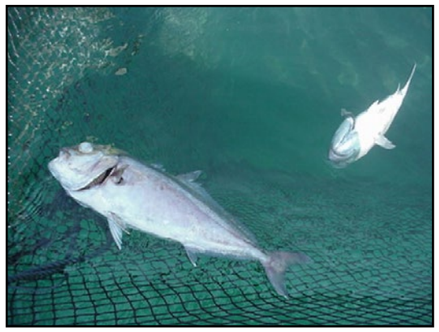


Figure 148. Streptococcosis infected greater amberjack (*Seriola dumerili*) which occurred one year post-stocking.

Clinical Signs

Typical but not pathognomonic signs of streptococcosis include cloudy eyes, exopthalmos and focal, ulcerative skin lesions along the caudal peduncle area. Necropsy findings include pale gills, liver haemorrhage, spleen congestion, kidney haemorrhage and liquefactive necrosis. There is not the generalised external haemorrhage or congestion as seen in other bacteraemias, but the haemorrhage is more organ specific in streptococcosis. Pericarditis is also observed (Figs. 149-158).

Figure 149. Greater amberjack (*Seriola dumerili*) with ocular cloudiness and ulcerated tail peduncle due to streptococcosis.

Figure 150. Corneal opacity with isolation of *Streptococcus morbillorum* in Greater amberjack (*Seriola dumerili*).

Figure 151. Progression of corneal opacity to corneal ulceration from streptococcosis in greater amberjack (*Seriola dumerili*); *S. morbillorum* isolated from an eye lesion and Lancefield group C *Streptococcus* isolated from a tail lesion.

Figure 152 Corneal perforation from streptococcosis in greater amberjack (*Seriola dumerili*).

Figure 153. Focal and deep haemorrhagic ulcerations from streptococcosis in greater amberjack (*Seriola dumerili*).

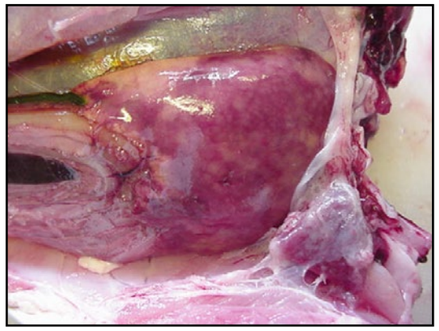


Figure 154. Liver haemorrhage and congestion from streptococcosis in greater amberjack (*Seriola dumerili*).

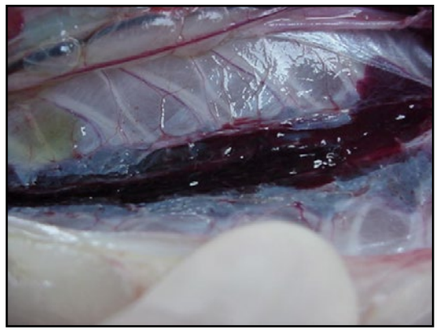


Figure 155. Haemorrhagic, liquefied kidney from streptococcosis in greater amberjack (*Seriola dumerili*).

Figure 156. Mangrove snapper (*Lutjanus argimenticulatus*) with exopthalmos and streptococcosis due to *Streptococcus* Lancefield group B.

Figure 157. Mangrove snapper (*Lutjanus argimenticulatus*) with panopthalmitis and collapsed ocular contents due to *Streptococcus* Lancefield group B infection.

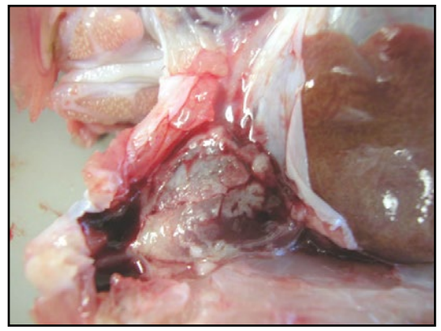


Figure 158. Giant grouper (*Epinephelus lanceolatus*) with severe pericarditis due to *Streptococcus acidominimus.*

Epidemiology

Streptococcosis occurs in the summer period with water temperatures of 28-29ºC. Generally streptococcosis identified in eight cases of fish disease caused mean mortalities of 38% (s=23) with a range of 3-70%.

In *Seriola dumerili*, cummulative mortality over a period of 3-4 weeks reportedly reached 70% with an acute mortality rate of 15-20%. Infected amberjack are of 1-1.5 kg having been well acclimatised 12 months after stocking. The fish are typically fed trash fish diets. Stocking density per cage is between 1,000 and 2,000 fish (considered moderate density). Spread between cages does occur particulary if infected cages of fish are towed to an area with uninfected fish. Sources of streptococci include the seabed sediment, contaminated trash fish feed and wildfish species (Inglis *et al*., 1993). Fish may be infected through skin, gill or eye abrasions caused by ectoparasites such as brooklynella, trichodina and benedinia. In Japan, *Streptococcus* species have been shown to survive in frozen fish diets for up to 6 months (Inglis *et al.*, 1993). Therefore the risk of streptococcosis is probably higher when wet diets such as trash fish are normally used.

Figure 159. *Benedinia* monogenean flukes on the skin of greater amberjack (*Seriola dumerili*), an example of co-parasitism which may stress fish leading to clinical streptococcosis.

Pathophysiology

Streptoccosis is a septicaemic condition. Bacteria may gain entry through the gills, skin and gut. Once in the host tissues, haematogenous spread occurs with localisation of infected thrombi in tissues such as kidney, spleen, eye, liver and heart (Figs. 161, 164 and 167).

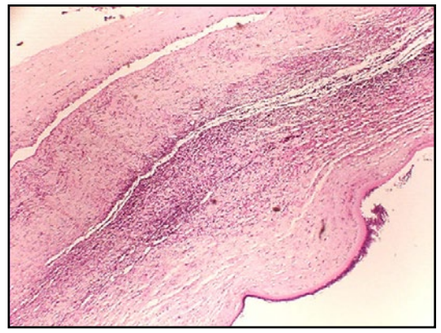


Figure 160. Diffuse keratitis from streptococcosis in greater amberjack (*Seriola dumerili*); *Streptococcus morbillorum* was isolated from the eye.

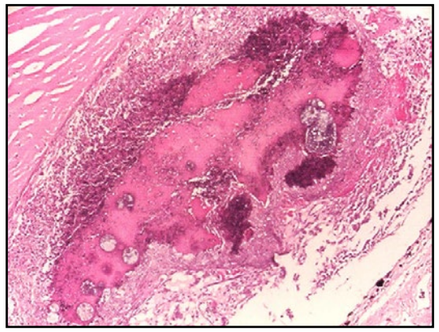


Figure 161. Infected thrombus in the anterior chamber of greater amberjack (*Seriola dumerili*) due to streptococcosis; *Streptococcus morbillorum* was isolated from the eye.

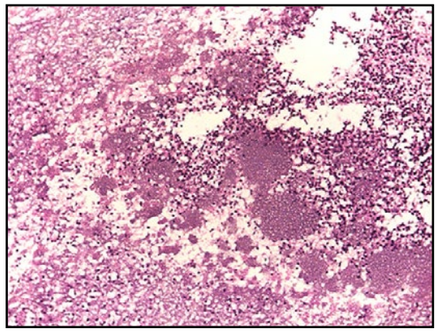


Figure 162. Bacterial colonies in the exudate of the eye; streptococcosis in greater amberjack (*Seriola dumerili*); *S. morbillorum* was isolated from the eye.

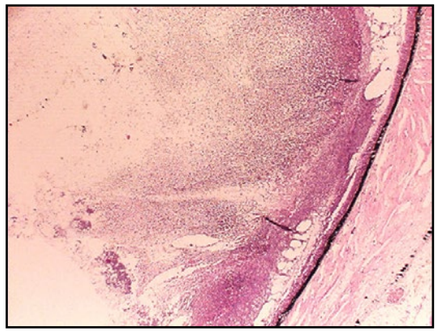


Figure 163. Septic exudate in the posterior chamber of the eye; streptococcosis in greater amberjack (*Seriola dumerili*)

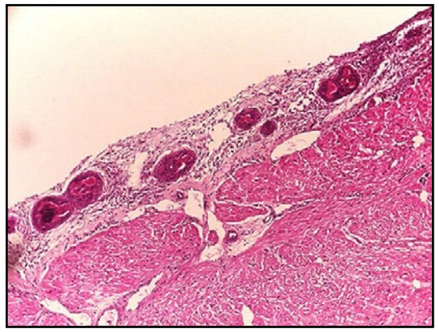


Figure 164. Septic thrombi in the pericardium from streptococcosis in greater amberjack (*Seriola dumerili*).

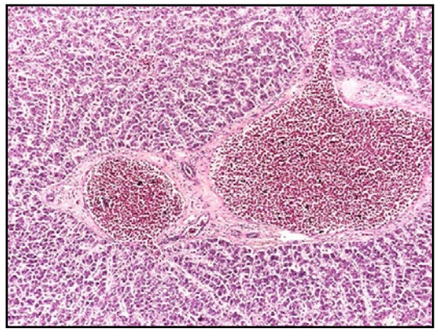


Figure 165. Liver congestion; streptococcosis in greater amberjack (*Seriola dumerili*).

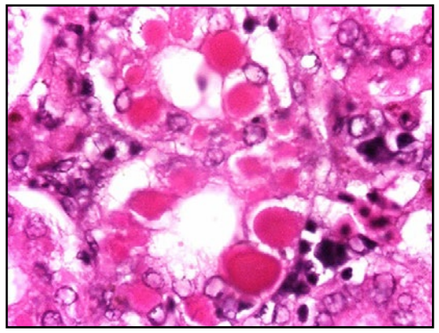


Figure 166. Renal tubular degeneration; streptococcosis in greater amberjack (*Seriola dumerili*).

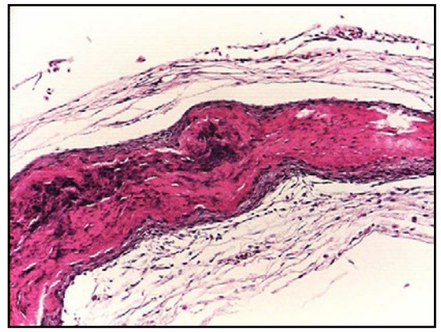


Figure 167. Fibrosing thrombus in the pericardial vessel; streptococcosis in greater amberjack (*Seriola dumerili*).

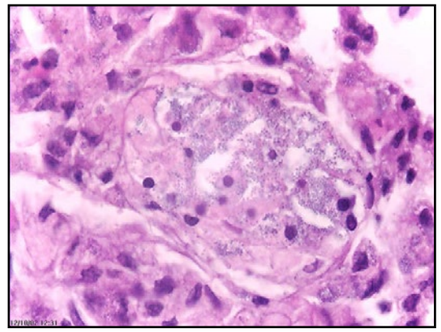


Figure 168. Giant grouper (*Epinephelus lanceolatus*) with *Streptococcus acidominimus* infection; bacterial cocci in macrophages of pericardium.

The septic emboli and thrombi are likely to occlude blood vessels particularly when they become organised and fibrosed. The time taken for thromboembolism may occur during the chronic course of the disease. In published reports of streptococcosis in fish, infection extended to the brain, resulting in swimming abnormalities. This however was not observed in local cases of streptococcosis although there was vascular congestion of the meninges. Colonies of coccoid bacteria in exudates of the eye, renal eosinophilic tubular epithelial degeneration and hepatic congestion were observed histological changes (Figs. 162, 163, 165, 166 and 168).

Diagnosis

Impression smears of lesions and Gram staining yielding Gram-positive cocci (Fig. 169) or macrophage phagocytosis of cocci (Fig. 168) provide a presumptive diagnosis. Cultures of kidney, heart, eye, spleen and lesions on blood agars with incubation at 25ºC or room temperature should yield round, white colonies which may become haemolytic in 24-48 hours. Colonies may be alpha-haemolytic meaning that they consume the iron in the haemoglobin in the blood agar plate producing a green discolouration. Beta-haemolytic colonies cause rupture of red cells on the blood agar plate producing a clear zone around the colonies. Biochemical profiles for streptococci include:

- Positive for - hydrolysis of esculin, decarboxylation of salicin and trehalose
- Negative for – hydrolysis of gelatin and decarboxylation of arabinose

Identification of *Streptococcus* spp. is based on the API 20 STREP test kit which uses 20 standardised biochemical tests to identify the most frequently encountered streptococcus groups or species. The enzymatic tests are inoculated with a dense suspension of organisms, made from a pure culture, which is used to rehydrate the enzymatic substrates. The metabolic end products produced during the incubation period are either revealed through spontaneous coloured reactions or by the addition of reagents. The fermentation tests are inoculated with an enriched medium, which reconstitutes the sugar substrates. Fermentation of carbohydrates is detected by a shift in the pH indicator.

Streptococcosis in local amberjack is due to *Streptococcus equisimilis, S. mobillorum* and *Streptococcus* Lancefield group C. They are both alpha-haemolytic. *S. acidominimus* was isolated from *E. lanceolatus.* Lancefield group B *Streptococcus* was isolated from *Lutjanus argimenticulatus, L. malabaricus* and *Acanthophthalmus kuhli. Streptoccocus* spp. were isolated from *Rachycentron canadum, Trachinotus blochii, L. malabaricus* and *Acanthopsis choiorhynchus*. Lancefield group D *Streptococcus* was isolated from *Lujanus rivulatus.* There are considerable changes in the taxonomy of *Streptococcus* species which may be better defined with molecular phylogenetic studies.

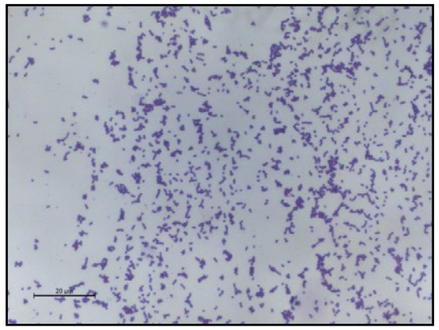


Figure 169. Greater amberjack (*Seriola dumerili*) with Gram positive cocci of *Streptoccocus* sp.

Antibiotic sensitivity results are:

Streptococcus mobillorum: sensitive to oxytetracycline, erythromycin, gentamicin, chloramphenicol, amoxycillin-clavulanic acid, enrofloxacin, doxycycline and minocycline; resistant to oxolinic acid, neomycin, trimethroprim -sulfamethoxazole and flumequine

Streptococcus Lancefield group C: sensitive to oxytetracycline, erythromycin, amoxycillin-clavulanic acid, enrofloxacin, trimethroprim-sulfamethoxazole, doxycycline and minocycline; resistant to oxolinic acid, neomycin, flumequine and gentamicin

Control and Prevention

Control of marine streptococcosis relies on the administration of an antibiotic to which the isolate is sensitive together with the control of any associated ectoparasites and improving husbandry practices. The protocol applied for these cases is :

1. Norfloxacin at 8 mg/kg fish once daily for 10 days in feed is prescribed. Disease mortalities are controlled at this dose regime. A withdrawal period of 30 days after the last day of antibiotic treatment for fish stocks is advised. Mortalities are controlled and losses in the treated fish are 30% compared to 70% in untreated fish.
2. Vitamin C at 1,000 mg/kg feed weight once daily is prescribed for ongoing prophylaxis to improve the immune response of fish.
3. Glucan at 0.5 kg/tonne of trash fish feed is prescribed for 3 weeks, followed by non-glucan feed for 3 weeks and then a final 3 weeks of Glucan as an immunostimulant.
4. Freshwater bathing of fish once a week for 3 weeks is advised to reduce the loading of *Brooklynella* and *Trichodina* parasites in the gills. This will also control *Benedinia* numbers on the fish.
5. To reduce the risk of streptococcosis, these husbandry options should be considered:
    a. Keep stocking density to 500-1,000 fish/ raft.
    b. Quarantine sick fish from healthy fish – keep sufficient separation as bacteria can be easily transmitted by water. Quarantine all new fish in a separate location. Do not mix batches of fish.
    c. Disinfect equipment, e.g., feed buckets, boats, clothing and footwear in 200 ppm chlorine solution on a regular basis to minimise transfer of bacteria. Ensure adequate rinsing to remove residue disinfectant before using equipment.
    d. Change to a commercial pelleted feed may reduce the risk of food-borne streptococcosis.

Preventative measures are dependent on an effective vaccine against the pathogenic strains of streptococci present in the fish environment or to which the susceptible fish stocks are likely to encounter.

Aeromoniasis

Farm History

Problems associated with aeromoniasis occur in freshwater pond fish farming. As described in the section on aquaculture systems, pond farming is polyculture with the continual mixing of batches of fish in a pond while partial harvesting occurs. This practice becomes problematic when a disease such as aeromoniasis occurs. Species affected include big head carp *(Hypophthalmichthys nobilis)*, tilapia (*Oreochromis* sp.) and mullet (*Mugilidae*).

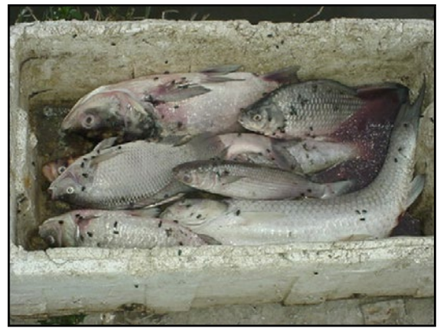


Figure 170. Mixed species of dead fish due to aeromoniasis.

Clinical Signs

Diseased fish exhibit severe haemorrhagic septicaemia. Gross pathology includes exopthalmos, ocular haemorrhage, scale loss and depigmentation, gill congestion, haemorrhagic kidney, splenomegaly and splenic necrosis. The liver is friable and oedematous. The intestine is fluid filled and congested. There is intra-cranial haemorrhage, swim bladder haemorrhage and ascites with blood-fluid in the abdominal cavity. External haemorrhages occur around the head and anal fin base (Figs. 171-177).

Figure 171. Big head carp *(Hypophthalmichthys nobilis)* with ocular haemorrhage and mild exopthalmos; aeromoniasis due to *A. sobria.*

Figure 172. Bighead carp *(Hypophthalmichthys nobilis)* with scale loss and skin depigmentation; aeromoniasis due to *A. sobria.*

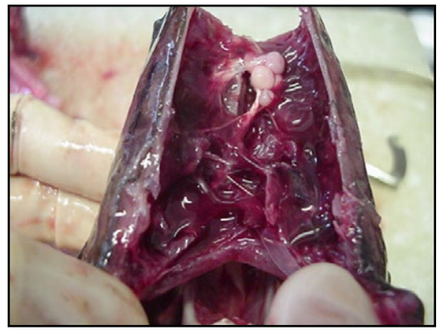


Figure 173. Big head carp *(Hypophthalmichthys nobilis)* with severe intracranial haemorrhaging; aeromoniasis due to *A. sobria.*

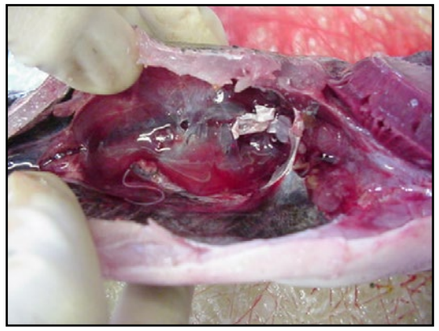


Figure 174. Swim bladder haemorrhage; aeromoniasis in big head carp *(Hypophthalmichthys nobilis)*; aeromoniasis due to *A. sobria.*

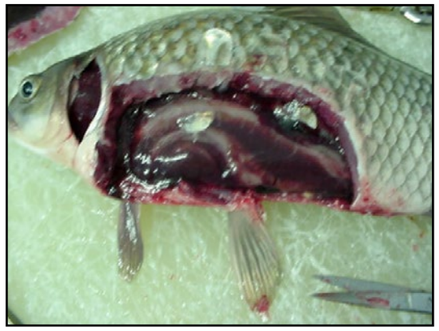


Figure 177. Grass carp (*Ctenopharyngodon idella*) with haemorrhagic viscera with aeromoniasis due to *A. sobria.*

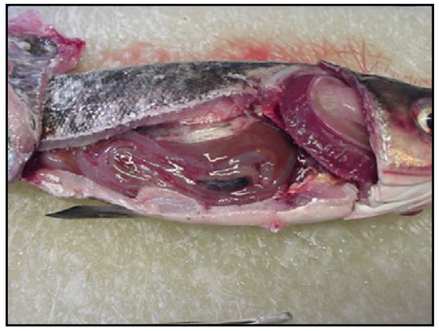


Figure 175. Gill and intestinal congestion; aeromoniasis in big head carp *(Hypophthalmichthys nobilis)*; aeromoniasis due to *A. sobria.*

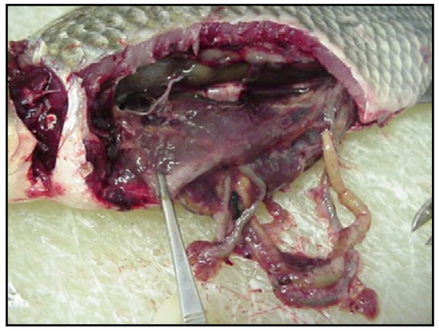


Figure 178. Friable, oedematous liver, haemorrhagic kidney and fluid filled intestines; aeromoniasis due to *A. sobria* in grass carp (*Ctenopharyngodon idella*).

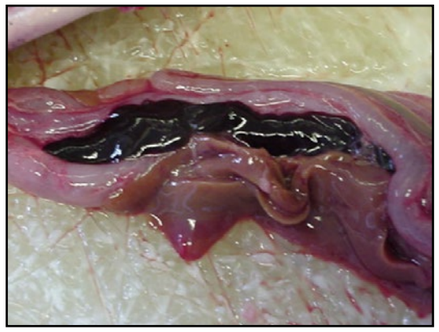


Figure 176. Splenomegaly and congestion; aeromoniasis due to *A. sobria* in big head carp *(Hypophthalmichthys nobilis)*.

Epidemiology

As *Aeromonas* spp. are part of the environmental flora, disease outbreaks are the culmination of poor water quality from the build-up of organic waste in the fish pond. This is exacerbated by high stocking density, poor water exchange, warm water temperatures and excessive feeding. Heavy ectoparasitism can also stress fish, leading to outbreaks. Mortalities at the pond level can be slow in onset but peak when pond conditions become more degraded.

Pathophysiology

Aeromonad septicaemia is reportedly due to the motile aeromonads *Aeromonas hydrophila, A. caviae* and *A. sobria.* In Hong Kong, *A. sobria* has been isolated from diseased freshwater fish affected by aeromoniasis. The predominant pathology of haemorrhage with secondary organ changes of congestion (Figs. 175-178) and oedema is suggestive of bacterial toxin induced damage (Fig. 179). For *A. hydrophila,* several toxins have been found, including gelatinase, lipase, elastase, haemolysins, cytotoxins and enterotoxins (Inglis *et al*., 1993).

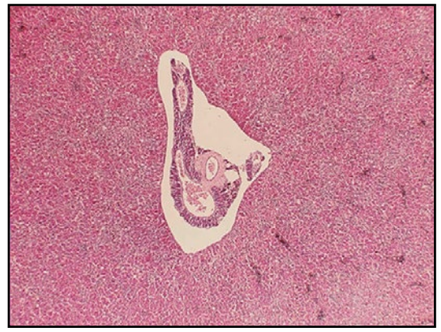


Figure 179. Bighead carp *(Hypophthalmichthys nobilis)* with splenic congestion and oedema of the exocrine pancreatic islet.

Diagnosis

Culture of *Aeromonas* spp. is carried out using kidney, spleen and liver tissues. Incubation on non-selective nutrient such as trypticase soy agar (TSA) at 25ºC for 24 hours produces white, circular, convex colonies. Gram-negative rods with motility are observed. The biochemical differentiation of A. *hydrophila* and *A. sobria* are shown in Table 31.

Table 31. Differentiation of *A. sobria* and *A. hydrophila*

Biochemical chracteritics	***A. hydrophila***	***A. sobria***
Aesculin hydrolysis	+	-
Growth in KCN broth	+	-
L-Histidine & L-Arginine utilisation	+	-
L-Arabinose utilisation	+	-
Fermentation of salicin	+	-

The antibiogram of two isolates of *A. sobria* are shown in Table 32.

Table 32. Antibiogram of *A. sobria*

Antibiogram	**S = sensitive R = resistant B = borderline**										
	OXO	**OXY**	**ERY**	**GEN**	**NEO**	**AMC**	**ENR**	**TRI**	**DOX**	**FLU**	**MIN**
A. sobria	S	S	B	S	S	R	S	S	S	S	S
A. sobria	S	S	B	S	S	B	S	S	S	S	S

Antibiotic Key : OXO oxolinic acid
OXY oxytetracycline
ERY erythromycin
GEN gentamicin
NEO neomycin
MIN minocycline
DOX doxycycline
ENR enrofloxacin
TRI trimethoprim-sulfamethoxazole
FLU flumequine
AMC amoxycillin clavulanic acid

Control and Prevention

Improving the culture environment and control of existing parasitic conditions such as gill trichodiniasis are important considerations in pond culture. Monitoring of water quality on a regular basis to check for dissolved oxygen, ammonia and suspended solids is important. When the DO is reduced, or when the ammonia and suspended solids increase, the water quality declines. Thus measures to increase water exchange rates and aeration, reduce feed rates or cap stocking density should be initiated. Without this monitoring and pond management, the pond environment can degrade to the extent that fish become very stressed and opportunistic bacteria such as *Aeromonas* spp. can initiate disease outbreaks.

Management of disease due to *Aeromonas* spp. with chemicals such as Dipterex (Trichorphon), bleach or lime applied to the water with fish in it is inappropriate. These treatments could exacerbate the mortalities. Fish that have died in the pond would be a source of nutrients for bacteria as well as increase the oxygen demand of the pond. Hence dead fish must be promptly removed from the pond.

Antibiotic therapy is only feasible in the very early course of the disease while fish are still eating and when the pond environment is not too degraded. Once the disease has spread in the pond the only viable options are to:

- destock the affected pond
- dry out and lime the pond bottom
- idle the affected pond for 3 months before restocking

To prevent pond to pond spread of disease:

- fish should not be mixed from different ponds.
- water should not flow from pond to pond.
- utensils and equipment should be dedicated to a pond or cleaned before use between ponds.

As aeromoniasis is a particular risk in relation to polyculture, care is needed when purchasing and stocking different species of fish, which may carry more virulent strains of aeromonads.

Pasteurellosis

Farm History

Marine pasteurellosis has been reported in cobia (*Rachycentron canadum*) fingerlings (Figs. 180-181). Imported fingerlings appear healthy on arrival but in the first 2 weeks post-stocking mortalities begin and become marked over 2-3 weeks. The disease does not appear to spread readily to other species of marine fish (seabream and red snapper) held adjacent to infected cobia.

Figure 180. Cobia (*Rachycentron canadum*) fingerlings in a sea cage.

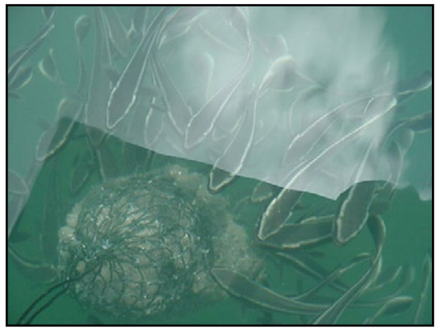


Figure 181. Cobia (*Rachycentron canadum*) fingerlings with pasteurellosis still appear to be eating floating pellets.

Clinical Signs

Pasteurellosis of cobia presents with no significant external lesions but it has been reported that some infected fish species develop haemorrhagic areas in the head and gills (Bergh, 2008). Infected fish appear thin, become increasingly emaciated and show clinical anaemia, e.g., pale gills and liver (Figs. 182-183). Necropsy findings include enlargement of spleen and kidney with white tubercles of 0.5-1.0 mm in diameter. Tubercles also appear in the liver (Figs. 183-186).

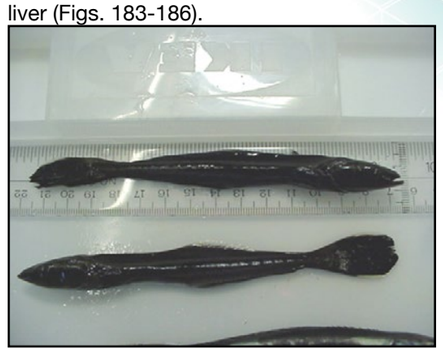


Figure 182. Emaciated cobia (*Rachycentron canadum*) with pasteurellosis.

Figure 183. Pale gills; cobia (*Rachycentron canadum*) with pasteurellosis.

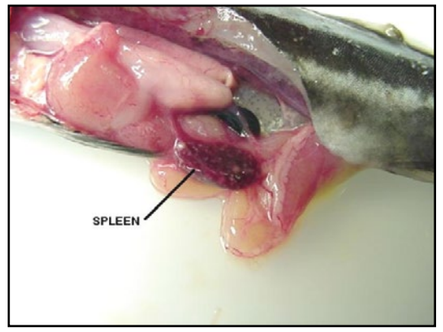


Figure 184. Spleen with white tubercles; cobia (*Rachycentron canadum*) with pasteurellosis.

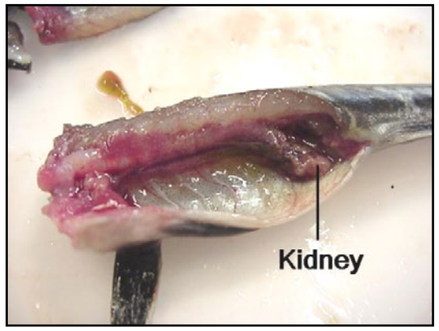


Figure 185. Kidney with white tubercles; cobia (*Rachycentron canadum*) with pasteurellosis.

Figure 186. Liver, spleen and kidney with white tubercles; note the kidney and spleen are almost completely filled with tubercles; cobia (*Rachycentron canadum*) with pasteurellosis.

Epidemiology

Pasteurellosis is not an acute disease. It is slow in onset with an incubation of 2 weeks. Infected fish progressively weaken and mortality rates gradually increase to 60% over 5-6 weeks. The stress of translocation may precipitate clinical disease in otherwise subclinical fish. Cobia appear to be more susceptible to clinical disease than seabream or red snapper as the co-habitating species do not develop the organ tubercles although *Photobacterium damsela* (previously *Pasteurella piscicida*) has been isolated from them. High temperatures (> 25°C) and high salinity (> 10 ppt) are favourable to infection (Stoskopf, 1993).

Pathophysiology

Photobacterium damsela may gain entry into fish via the gills or gut lining. There is no necrotising effect on these tissues but haematogeneous spread to the spleen and kidney occurs. The host organ attempts to contain the bacteria in the haematopoeitic tissues but where this is not successful, the infection extends to the liver. Granuloma formation (Figs. 187-189, 191) with surrounding large epithelioid macrophages is the typical host response, thus producing the grossly visible white tubercles. Some lesions are more acute and resemble abscesses (Fig. 190) with necrotic centres (Fig. 188) containing *P. damsela* (Fig. 192). Free colonies of bacteria may exist prior to granuloma formation and these are associated with tissue necrosis.

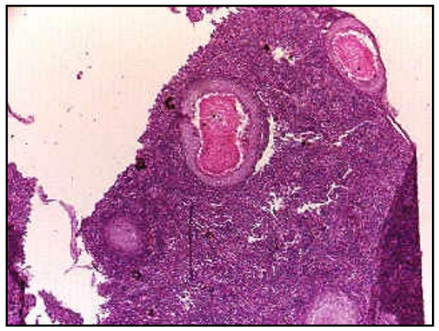


Figure 187. Splenic granulomas; cobia *(Rachycentron canadum)* with pasteurellosis.

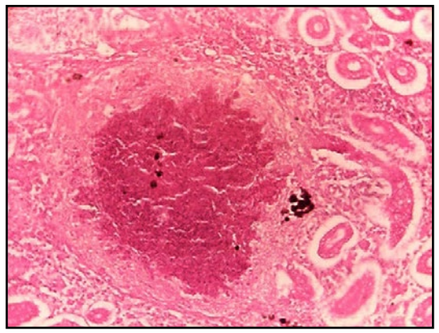


Figure 188. Kidney granuloma with necrotic centre; cobia *(Rachycentron canadum)* with pasteurellosis.

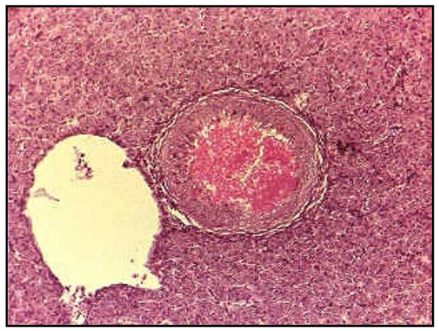


Figure 189. Liver granuloma; cobia *(Rachycentron canadum)* with pasteurellosis.

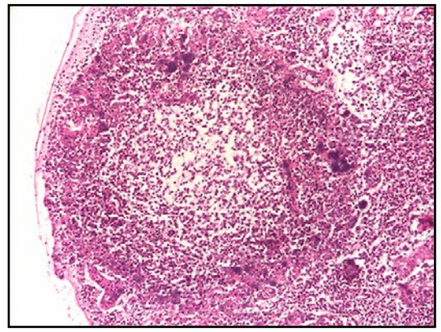


Figure 190. Splenic abscess with necrotic centre; cobia *(Rachycentron canadum)* with pasteurellosis.

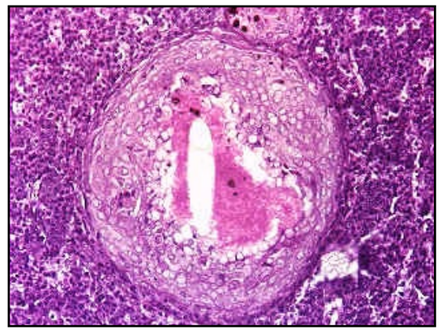


Figure 191. Kidney granuloma with prominent epithelioid macrophages forming the wall; cobia *(Rachycentron canadum)* with pasteurellosis.

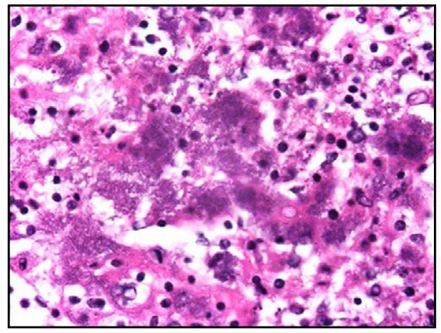


Figure 192. Bacteria and splenic necrosis; cobia *(Rachycentron canadum)* with pasteurellosis.

Diagnosis

Photobacterium damsela is readily cultured on nutrient media with 1-2% sodium chloride, following incubation for 48 hours at 25 °C. Gram staining of kidney or spleen containing white tubercles impression smears reveals Gram-negative plump rods that stain strongly on both ends (bipolar staining) (Fig. 193).

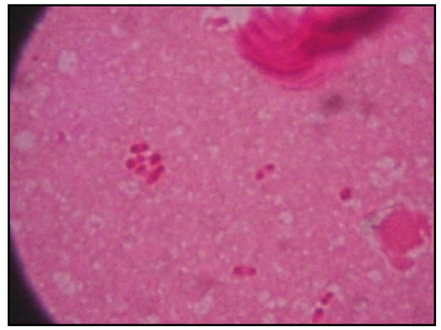


Figure 193. Cobia *(Rachycentron canadum)* Gram stain and kidney smear showing bipolar staining Gram negative plump rods.

The biochemical characteristics of *P. damsela* are listed in Table 33.

Table 33. Biochemical characteritics of *Photobacterium damsela* (adapted from Inglis *et al.* (1993))

Oxidase	+
Catalase	+
Indole production	-
Nitrate production	-
Acid from glucose, fructose, galactose and mannose	+
Decarboxylation of ornithine & lysine	-
Haemolysis	-
Acid from esculin and salicin	-

The antibiogram profile for *P. damsela* is given in Table 34.

Isolates from Cobia (C1-3), Seabream (SB 1-2) and Red snapper (RS 1-3)

Table 34. Antibiogram for *Photobacterium damsela* isolates

Antibiogram	S = sensitive R = resistant B = borderline										
	OXO	**OXY**	**ERY**	**GEN**	**NEO**	**AMC**	**ENR**	**TRI**	**DOX**	**FLU**	**MIN**
C1	**S**	R	B	S	S	S	S	R	R	S	B
C2	**S**	S	R	S	R	S	S	S	S	S	S
C3	**S**	S	B	S	B	S	S	S	S	S	S
SB1	**S**	S	B	S	B	S	S	S	S	S	S
SB2	**S**	S	B	S	B	S	S	S	S	S	S
RS1	**S**	S	R	S	B	S	S	S	S	S	S
RS2	**S**	S	B	B	B	S	S	S	S	S	S
RS3	**S**	S	R	S	R	S	S	S	S	S	S

Antibiotic Key:

OXO	oxolinic acid	DOX	doxycycline
OXY	oxytetracycline	ENR	enrofloxacin
ERY	erythromycin	TRI	trimethoprim-sulfamethoxazole
GEN	gentamicin	FLU	flumequine
NEO	neomycin	AMC	amoxycillin clavulanic acid
MIN	minocycline		

Control and Prevention

One of the difficulties with treating marine pasteurellosis is the presence of subclinical carrier fish – in this case the seabream and red snapper that are co-cultured on farms. This can lead to re-infection of the cobia and prolonged antibiotic treatment is required. This could increase the risk of antibiotic resistance development, hence the need to repeat fish sampling, bacterial isolations and antibiogram profiling to ensure that the antibiotic used is still effective against the isolates of *P. damsela.*

Generally, the disease can be managed with the goal of reducing the mortality rate and improving the body condition of the cobia. These include:

- Reducing the stocking density per sea cage from 500 to 200–300
- Frequent changing (every 2 days) of the finer mesh nets, which are easily fouled with sediment every 2 days
- Reduce daily feeding from 6 times daily to 3-4 times in order to reduce post-prandial oxygen demand and stress, as fish are anaemic.
- Freshwater bathing to reduce any gill protozoal parasites and to ameliorate osmotic stress
- Provision of 24-hour aeration to maintain dissolved oxygen above 5 ppm
- Amoxycillin-clavulanic acid (375 mg tablet) is prescribed to be administered at 50 mg/kg fish once daily in food for 10-30 days, depending on the response. The withholding period is 30 days for treated fish and 15 days for fish held in contact with treated fish.

Although marketed as a useful tool in disease management, immunostimulant glucan fails to benefit fish during the course of clinical pasteurellosis.
After 30 days of antibiotic treatment, fish respond to amoxycillin-clavulanic acid with the observation of reducing mortalities, improved body condition indices and localisation of bacterial lesions within matured granulomas with less free bacterial colonies that cause kidney, spleen or liver damage.

Commercial vaccination against *P. damsela* subsp. *pscicida* has recently become available. The efficacy of vaccination depends on the size of fish and the type of challenge. In seabass (*Dicentrarchus labrax*), vaccinated fish of size 1.5-2 g failed to be protected while protection was elicited in 20 g fish (Bakopoulos *et al*., 2003). Oral vaccination also afforded some protection. However, vaccinated fish were not protected when challenged by intraperitoneal injection.

Mycobacteriosis

Farm History

Mycobacteriosis is a disease particularly of freshwater ornamental fish in Hong Kong, although it can occur in brackish and marine fish species. Species observed to be infected with *Mycobacterium* spp. include goldfish (*Carassius auratus*), neon tetra (*Paracheirodon innesi*) and discus (*Symphysodon discus*). While acute losses are uncommon, the problems with mycobacteriosis is that it can preclude fish from export to certain countries and it is a zoonotic disease.

Clinical Signs

Mycobacteriosis causes a chronic wasting condition in heavily infected fish (Figs. 194-196). Nevertheless, subclinical carrier states are common and fish can appear normal. On necropsy, granulomatous lesions (Fig. 197) may be observed in the viscera, particularly spleen and liver, but also in the heart and pancreas. Severe infections result in fish being very thin and having generalised granuloma formation in the viscera.

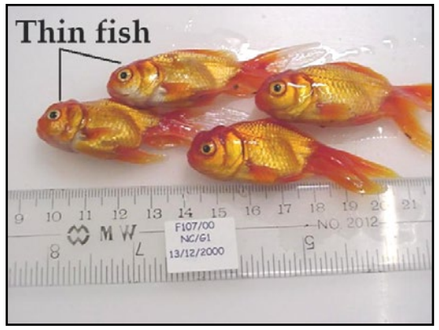


Figure 194. Red oranda goldfish (*Carassius auratus*) showing emaciation; note the sunken dorsal musculature. *Mycobacterium* sp. isolated.

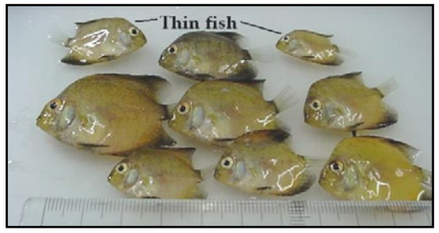


Figure 195. Discus (*Symphysodon discus*) with mycobacteriosis; note some thin fish in the batch.

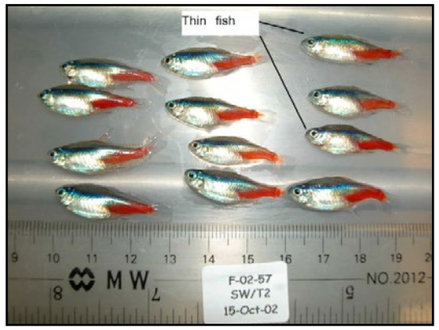


Figure 196. Neon tetra (*Paracheirodon innesi*) with mycobacteriosis; note some thin fish in the batch.

Figure 197. White granulomas in the spleen; red lion head goldfish (*Carassius auratus*) with mycobacteriosis.

Epidemiology

Mycobacteriosis in finfish is caused by *Mycobacterium fortuitum, M. chelonae* and *M. marinum*. These are ubiquitous organisms in the aquatic environment. Clinical disease occurs when fish are held in high stocking density with infected fish shedding bacterium into the water. Transmission is thought to occur primarily through cannibalism of dead infected fish carcasses.

Pathophysiology

Infection with *Mycobacterium* spp. typically elicits a granulomatous host response (Figs. 198-201), in the spleen and kidney haematopoeitic tissue. Granuloma formation is the host's attempt to contain the bacteria from spreading. It is quite common not to observe bacteria within the matured granulomas even with ZN staining (Figs. 202-203) probably as a result of host destruction of the bacteria. Nevertheless, the progressive loss of body condition could stem from the diversion of available metabolic energy from growth to the production of granulomas. Heavily infected fish may die from this negative energy balance or emaciation.

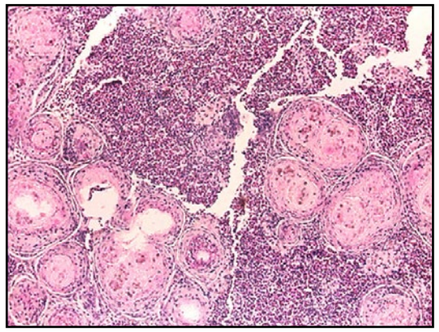


Figure 198. Multiple splenic granulomas; red oranda goldfish (*Carassius auratus*) with mycobacteriosis.

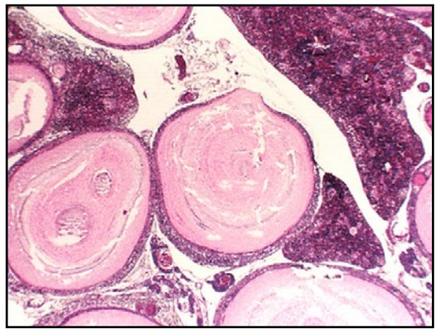


Figure 199. Multiple and large splenic granulomas; discus (*Symphysodon discus*) with mycobacteriosis.

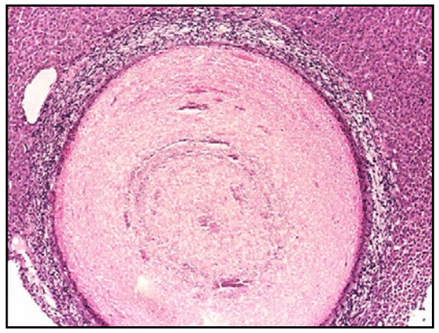


Figure 200. Very large granuloma with distinct layering in the liver; discus (*Symphysodon discus*) with mycobacteriosis.

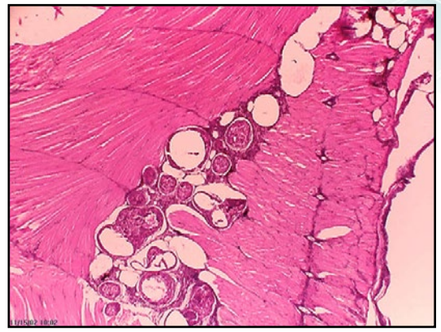


Figure 201. Muscle granulomas; neon tetra (*Paracheirodon innesi*) with mycobacteriosis.

Diagnosis

The diagnosis of mycobacteriosis is based on gross pathology, histopathology and culture isolation of *Mycobacterium* sp. One of the characteristics of *Mycobacterium* spp. is the acid-alcohol fastness of its cell wall. This allows the bacterium to resist decolourisation by acidified alcohol (Ziehl-Neelsen staining method). Therefore in tissue smears of granulomas where active infection is occurring, the ZN stain reveals mycobacteria as acid-fast (red-pink) rods. Mycobacteria are also weakly Gram-positive. Isolation of mycobacteria by culture requires the following:

- Lowenstein-Jensen media which contains anhydrous monopotassium phosphate, magnesium sulphate, magnesium citrate, asparagine, potato flour, glycerol, distilled water, homogenised whole eggs and 2% aqueous malachite green.
- Aerobic incubation at 20-30ºC for up to 30 days; *M. fortuitum* and *M. chelonae* colonies may grow in 7 days but *M. marinum* may take several weeks to grow.
- Note: mycobacteria are zoonotic bacteria and culture work should be carried out in a biosafety cabinet.

Culture of mycobacteria can be unsuccessful despite sampling from fish showing a large number of granulomas. This may be due to insufficient inoculum, the bacteria being inactivated by the host response or failure to externalise the bacteria from the granulomatous tissue onto the culture media. It is also possible to recover mycobacteria from fish but the fish do not exhibit granuloma formation, which is probably an indication that these organisms are ubiquitous in the aquatic environment. At the time of this work, selective culture was the routine method of diagnosis. Nowadays, molecular methods are applied for the detection and typing of

Mycobacterium spp. which markedly reduces the turnaround time for diagnosis. Mycobacteria PCR testing of formalin-fixed tissues is a recent method of diagnosis (Pourahmad *et al*., 2009).

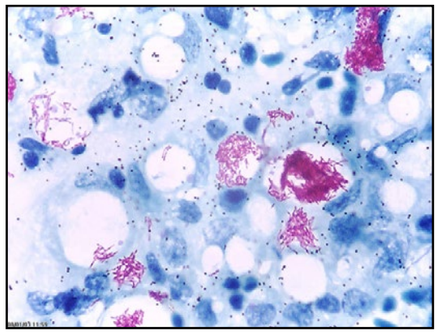


Figure 202. Ziehl-Neelsen stain showing acid-fast rods in a kidney smear of neon tetra *(Paracheirodon innesi)* with mycobacteriosis.

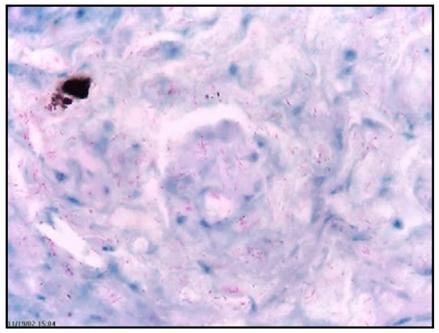


Figure 203. Ziehl-Neelsen stain showing acid-fast rods in granuloma; neon tetra *(Paracheirodon innesi)* with mycobacteriosis.

Culture characteristics of *M. marinum, M. fortuitum* and *M. chelonae* are listed in Table 35.

Control and Prevention

In Hong Kong, detection of mycobacteriosis in batches of ornamental fish leads to exclusion from export and a recommendation that the farm or facility cull these fish. This is aimed at reducing the spread of the disease between batches of fish, reducing the exposure risk of fish farm workers to zoonosis from the bacterium and improving the quality of fish exported.

No antibiotic therapy is recommended in the management of mycobacteriosis in ornamenantal fish. The reasons for this are:

- *Mycobacterium* spp. are notably multi-resistant to antibiotics (Aubry, 2000).
- Being ubiquitous, re-infection through cannibalism of carrier fish in a batch is problematic.
- Environmental hygiene and good husbandry (lower stocking density) are the key to avoiding clinical outbreaks of mycobacteriosis.

Following culling, disinfection of the tanks and utensils in chlorine bleach is indicated.

Table 35. Characteristics of mycobacteria in fish (adapted from Inglis *et al*. (1993))

Feature	*M. marinum*	*M. fortuitum*	*M. chelonae*
Isolation at 37 °C	-	+	-
Growth rate	slow	fast	fast
Pigmentation	photochromogenic	-	-
Growth on MacConkey	-	+	+
Nitrate reduction	-	+	-

Nocardiosis

Farm History

Nocardiosis is a disease of marine fish affecting species such as pompano. It is a chronic disease (3-4 months) and may not be immediately obvious to the farmer, although cumulative mortalities can reach 30-40%.

Clinical Signs

The gross pathology in one case of nocardiosis in pompano showed an ulcer in the midline of the body with abnormal bulging on both sides. A large mass was dissected which contained whitish caseous (cheesy) material similar to that of an abscess. The abscess extended through the muscles and spine. A small circular skin ulcer on the ventral body near the pelvic fins was also seen. Figs. 204-208 illustrate the clinical signs and gross pathology of this case.

Figure 204. Pompano (*Trachinotus blochii*) with central bulge at the level of the lateral line.

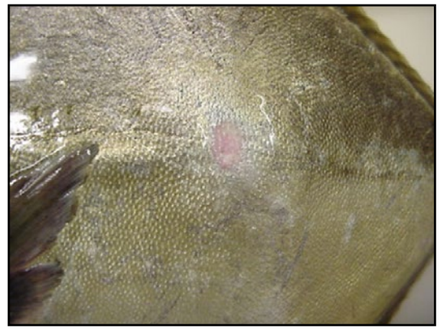


Figure 205. Pompano (*Trachinotus blochii*) with ulcerated skin area over the internal abscess; compression by the abscess with loss of blood supply to the overlying tissue may result in rupture through to the exterior.

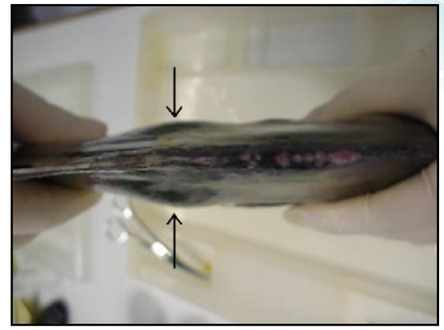


Figure 206. Extension of the body bulge on both sides of the flank (arrows); pompano (*Trachinotus blochii*).

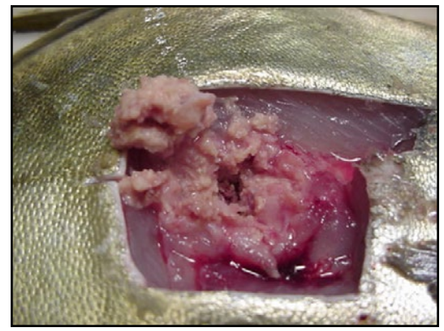


Figure 207. Pompano (*Trachinotus blochii*) with a cheesy (caseous) abscess through muscle.

Figure 208. Nocardial abscess perforated through the other side of pompano (*Trachinotus blochii*).

Epidemiology

Nocardiosis is a chronic infection leading to debility in infected fish stocks rather than producing acute losses. Nevertheless incremental fish mortalities do occur as infected fish become increasingly injured by the chronic inflammation which the bacterium induces. Nocardiosis in freshwater fish is caused by *Nocardia asteroides* while *Norcadia kampachi* causes marine infections in yellowtail (Roberts,

2001). *Nocardia seriolae* causes disease in *Seriola quinqueradiata* (yellowtail) in Japan (Itano *et al.*, 2006). In this species, nocardiosis-related fish mortalities are more common in autumn and winter months which may be related to the decline in the fish immune responses (Sheppard, 2004).

Pathophysiology

Nocardia sp. initiates a granulomatous host response in the kidney and spleen. However the bacterium is locally invasive and will extend into adjacent tissues such as muscle and bone. In this case, infection of the spine is observed. There is caseous inflammation consisting of necrotic tissue. Figures 209-214 illustrate the histopathology of nocardiosis.

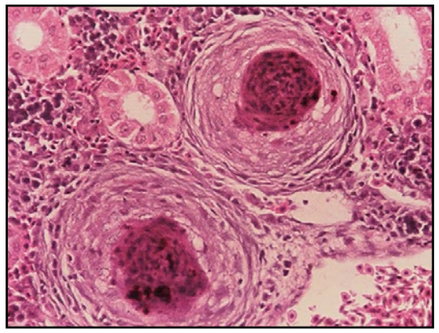


Figure 209. Renal granulomas - mature; pompano (*Trachinotus blochii*) with nocardiosis.

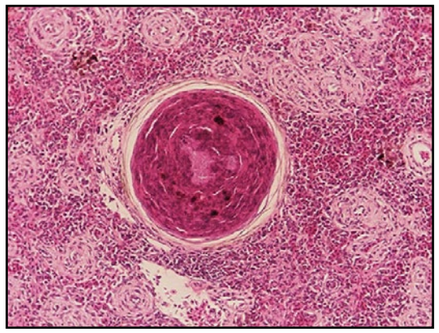


Figure 210. Splenic granuloma – mature; pompano (*Trachinotus blochii*) with nocardiosis.

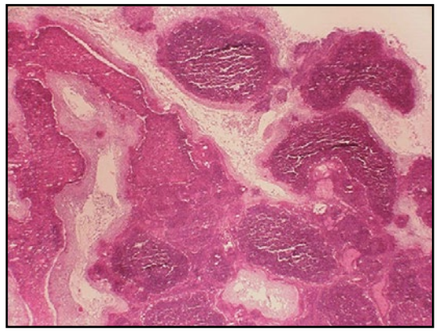


Figure 211. Granulomas extending into muscle and connective tissues; pompano (*Trachinotus blochii*) with nocardiosis.

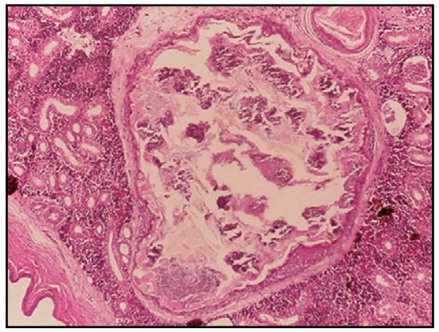


Figure 212. Renal granuloma – active with bacterial colonies in the centre; pompano (*Trachinotus blochii*) with nocardiosis.

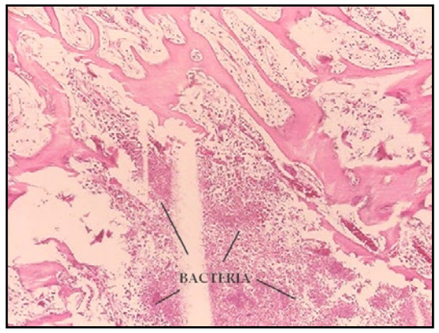


Figure 213. Pompano (*Trachinotus blochii*) spine; *Nocardia* sp. extending into the spinal bones, causing bony destruction and inflammation.

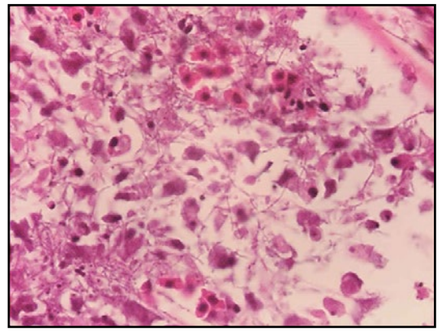


Figure 214. *Nocardia* sp. seen as branching filaments and associated necrotic inflammatory exudate; pompano (*Trachinotus blochii*).

Diagnosis

Bacterial culture may be performed using blood agar and brain heart infusion agar (BHIA) for *N. asteroides* (Buller, 2004), while sodium chloride is added to nutrient agar for *N. kampachi.* Lowenstein-Jensen media may also be used. Incubation is at 25-30ºC for 14 days. *N. asteroides* colonies are ridged, irregular, pink-white to yellow orange with aerial hyphae around the edges. *N. kampachi* colonies are flat and wrinkled (Inglis *et al*., 1993). Biochemical reactions for *N. asteroides* include:

- Acid production from glucose and glycerol but negative for arabinose, inositol, lactose and maltose.
- Carbon source from acetate, malate and propionate but not from benzoate.

N. kampachi is biochemically very similar to *N. asteroides* except that it does not grow at 37ºC (Inglis *et al*., 1993; Roberts, 2001).

Nocardia spp. staining characteristics are Gram-positive and acid-fast. The bacterium is a branching filamentous rod.

Control and Prevention

Control of nocardiosis with antibiotics is difficult due to its chronic course of infection. Attention to good husbandry through reduced stocking density is necessary.

Flavobacteriosis

Farm History

Marine farms experience fish mortalities from flavobacteriosis (previously flexibacteriosis) commonly and in association with outbreaks of vibriosis. Usually juveniles are most susceptible and species affected include grouper, s eabream and snapper. Losses are severe if fish are treated late in the disease, resulting in mortalities greater than 50%.

Clinical Signs

Although not pathognomonic, tail and fin erosions with ascending necrosis of the caudal peduncle is commonly observed in flavobacteriosis. Skin erosions can lead to extensive ulcerations although these are not very deep (Figs. 215, 220 and 221). The centre of the lesions are usually non-haemorrhagic although there may be erythema of the advancing edge. Despite extensive ascending tail lesions where the bony stump of the caudal vertebral column has been exposed (Figs. 216-219), infected fish still actively swim and show little sign of toxaemia or septicaemia. The classical saddle back lesion (Fig. 222) is occasionally observed in marine flavobacteriosis in Hong Kong. Gill necrosis (Figs. 223-224 and 228) can also be observed with masses of filamentous bacteria.

Figure 215. Extensive erosions of the pectoral and pelvic fins; flavobacteriosis in green grouper (*Epinephelus coioides*).

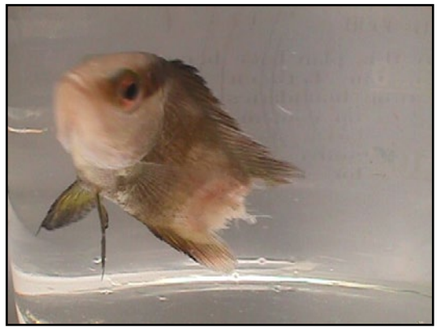


Figure 216. Red snapper (*Lutjanus malabaricus*) fingerling with completely eroded caudal peduncle, exposing the caudal vertebral column.

Figure 217. Green grouper (*Epinephelus coioides*) with necrotizing caudal peduncle.

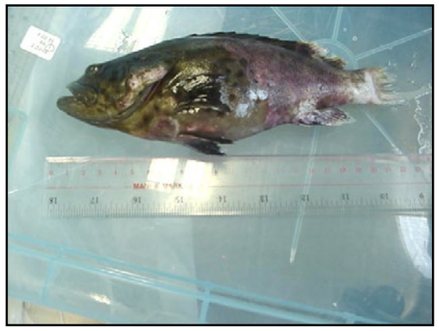


Figure 218. Note the advancing lesion edge of the necrotising tail rot. Green grouper (*Epinephelus coioides*) with mixed vibriosis and flavobacteriosis.

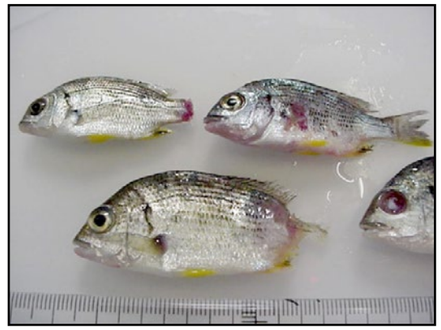


Figure 219. Seabream (*Pragus* sp.) fingerlings with lesions typical of flavobacteriosis and vibriosis.

Figure 220. Red snapper (*Lutjanus malabaricus*) with secondary flavobacteriosis in association with crytocaryoniasis and vibriosis.

Figure 221. Severe musculoskeletal necrosis; flavobacteriosis, vibriosis and cryptocaryoniasis in Red snapper (*Lutjanus malabaricus*).

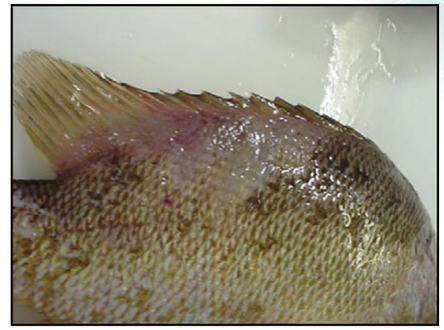


Figure 222. Red snapper (*Lutjanus malabaricus*) with a ‘saddle back lesion’ associated with *Flavobacter* sp. and uronema protozoa.

Figure 223. Red snapper (*Lutjanus malabaricus*) with gill necrosis associated with *Flavobacter* sp.

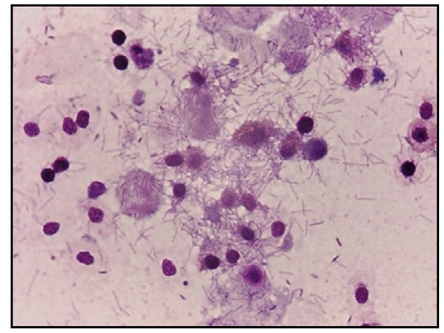


Figure 224. Giemsa stained smear of a gill lesion showing filamentous bacteria; red snapper (*Lutjanus malabaricus*).

Epidemiology

Flavobacteriosis is typically a secondary bacterial disease that follows after and aggravates lesions caused by ectoparasites and vibriosis. The disease is problematic in marine farms at water temperatures near 30ºC . The literature reports that the prevalence and severity of the disease is increased above 15 ºC (Wakabayashi, 1993; Toranzo *et al*., 2005). Therefore it can be difficult to assign the primary cause of an outbreak. Usually there has been some recent handling of the fish either through grading or moving fish between rafts. The onset of tail rot is fairly swift and can spread across a batch of fish within 1-2 days. Significant mortalities are probably related to the concurrent onset of vibriosis.

Pathophysiology

Flavobacteriosis is caused by *Tenacibaculum maritimus* (previously *Flexibacter maritimus*) in marine fish and *Flavobacterium columnare* (previously *Flexibacter columnaris*) in freshwater fish. Infected fish are not observed to develop septicaemia despite very severe necrotising activity in the skin (Figs. 225-226), muscle and bony structures of fish. The cause of mortality if solely due to flexibacteriosis may be due to osmoregulatory failure from exposed integument and toxic effects from endotoxins released by necrotic tissue.

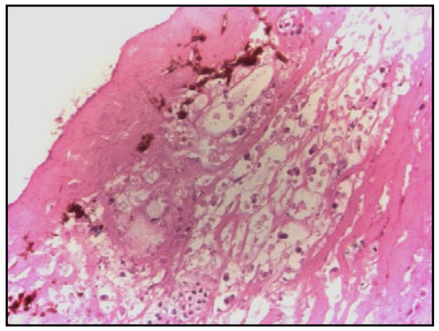


Figure 225. Subdermal oedema with erosion of the epidermis and scales; filamentous bacteria in the exposed dermis; red snapper *(Lutjanus malabaricus)*.

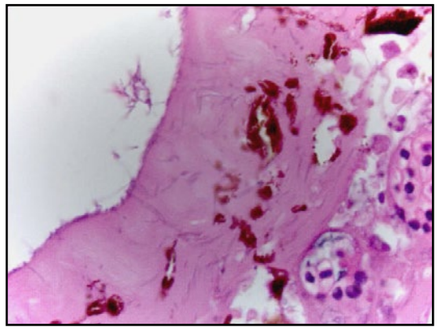


Figure 226. Filamentous bacteria invading the dermal layer of skin; red snapper *(Lutjanus malabaricus)*.

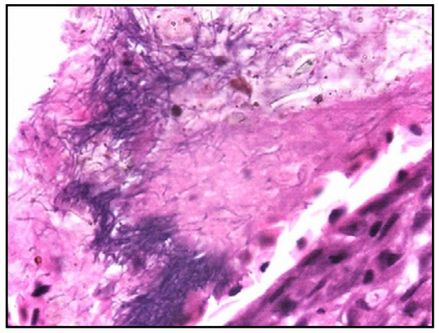


Figure 227. Gill necrosis due to flavobacteriosis; note the masses of filamentous bacteria in the gill exudates. Red snapper *(Lutjanus malabaricus)*.

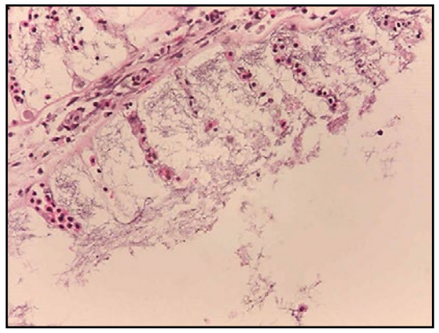


Figure 228. Necrotising flavobacteriosis of the gills; note the tendency of the bacteria to stack up in the interlamellar space; red snapper *(Lutjanus malabaricus)*.

Diagnosis

Typically diagnosis is from wet preparations and smears of lesions to demonstrate the presence of Gram-negative filamentous rods (Fig. 229). Culture may be unsuccessful due to the overgrowth of pathogenic vibrios in the sample. *Flexibacter* sp., *Tenacibaculum maritinum, Cytophaga* sp. and *Flavobacterium* sp. may be cultured on Anacker-Ordal (AO) agar which consists of bacto-tryptone, yeast extract, sodium acetate, beef extract, agar and distilled water. With marine samples, artificial seasalts should be added or sterilised seawater used at a 50-100% final concentration (Buller, 2004). Incubation is at 25ºC for 2-5 days. *T. maritinum* colonies are pale yellow to orange, flat, thin irregular colonies with ragged edges that adhere firmly to the agar. The biochemical reactions are as follows:

- Production of catalase, cytochrome oxidase and ammonium
- Hydrolysis of casein, gelatin, tributyrin and tyrosin
- Utilisation of tryptone, yeast extract and casamino acids
- Inability to degrade cellulose, chitin, starch and aesculin
- Inability to produce acid from glucose, galactose, fructose, mannose, lactose, sucrose, sorbose, maltose, cellobiose or trehalose

Antibiogram profiles are in Table 36 :

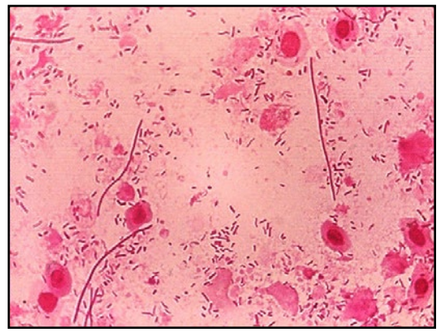


Figure 229. *Flavobacter* sp. and Vibrios from a Gram stained smear of a skin lesion; giant grouper *(Epinephelus lanceolatus)*.

Table 36. Antibiogram profiles of *Flexibacter* and *Flavobacterium* spp.

Bacterium sp.	Sensitive	Resistant
Flexibacter sp.	erythromycin, amoxycillin-clavulanic acid, trimethoprim-sulphamethoxazole	gentamicin doxycycline
Flavobacterium sp.	oxytetracycline, gentamicin, neomycin, erythro-mycin , chloramphenicol, amoxycillin-clavulanic acid, kanamycin, trimethoprim-sulphamethoxazole, flumequine, minocycline	oxolinic acid

Control and Prevention

Treatment of flavobacteriosis is dependent on the following principles:

- Removal of severely necrotised fish so as to reduce the release of large numbers of bacteria into the water column.
- Control of vibriosis – this means that the choice of antibiotic (Fig. 231) must be one to which both isolated vibriosis and flavobacteria are sensitive to.
- Control of ectoparasites such as *Cryptocaryon, Brooklynella* and *Trichodina* in marine fish.

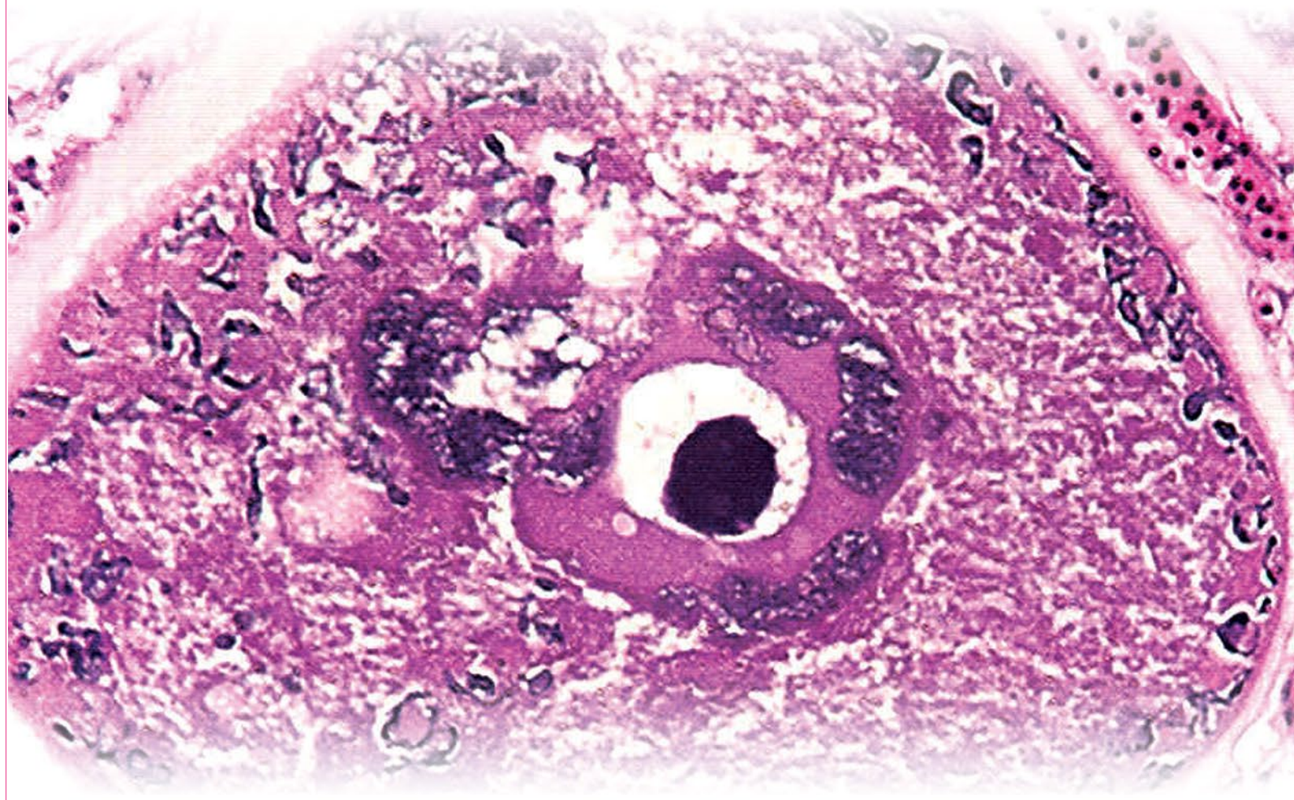
Preventive measures rely on:

- Reduction of stocking density to optimal levels commensurate with the rates of water exchange, volume of the pen and sizes of the fish (Fig. 230).
- Handling methods that minimise fin, tail and body abrasions, for example using knotless nets.
- Control of ectoparasites through regular checks and freshwater bathing when the numbers of parasites are increasing.
- In Spain, a bacterin is used to vaccinate turbot against mortalities caused by *T. maritimus* (Santos *et al*., 1999).

Figure 230. Crowding behaviour of grouper (*Epinephelus* sp.) may increase the risk of body abrasions which are the precursor to flexibacteriosis.

Figure 231. Preparing a tarpaulin for freshwater, formalin or antibiotic bathing.

PART 4 – VIRAL DISEASES



PART 4 – VIRAL DISEASES

Viral Encephalopathy and Retinopathy

Farm history

Viral encephalopathy and retinopathy (VER) is a viral disease of hatchery fingerlings affecting marine species. Clinical disease has been detected in imported *Cromileptes altivelis* (humpback grouper). Non-clinical infections have been detected in imported *Epinephelus coioides* (green grouper), *Epinephelus fuscoguttatus* (tiger grouper) and locally caught *Rhabdosargus sarba* (golden-lined seabream). VER or Viral Nervous Necrosis (VNN) is caused by related viruses of the Nodaviridae. In Hong Kong, the Red Spotted Grouper Nervous Necrosis Virus (RGNNV) genotype is involved with VER in clinical outbreaks.

Clinical signs

In the case of C. *altivelis*, larger fingerlings (6.7-7.3 cm in length) cannot swim properly and remain at the bottom of tanks initially (Fig. 232). After 24 hours, they lie on their sides, but beware that this is not specific for nodavirus disease. Fish may twist their bodies in an attempt to swim (Figs. 233-234) but gradually these infected fish become thin and die after a week of the onset of symptoms. On necropsy, the swim bladder is under inflated, and the spleen is enlarged and congested (Fig. 235).

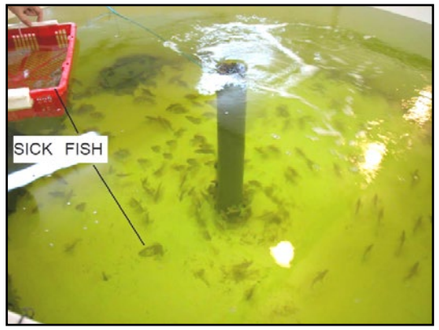


Figure 232. Recirculation system tank with sick humpback grouper (*Cromileptes altivelis*) unable to maintain posture in the water column; some sick fish placed in a floating basket for observation.

Figure 233. Normal humpback grouper (*Cromileptes altivelis*) able to maintain balance.

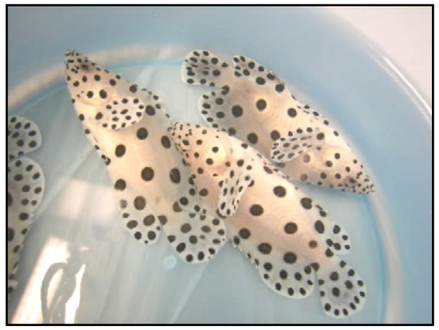


Figure 234. VER affected humpback grouper (*Cromileptes altivelis*) lying on their sides.

Figure 235. VER affected humpback grouper (*Cromileptes altivelis*) with enlarged spleen.

Epidemiology

Fish imported into Hong Kong may well be infected with nodavirus. Upon stocking and a change in the holding environment, stressors of translocation may favour the development of clinical VER in previously subclinical fish. In the first month of stocking, fish begin showing symptoms of VER. The reported cummulative mortality is 15%.

Pathophysiology

The pathogenicity of nodavirus in *C. altivelis* is specific to cells of the brain and retina, causing focal degeneration and vacuolation (Figs. 236-239). Mortality in hatchery sized fish would appear to be associated with failure to feed properly due to swimbladder and swimming abnormalities. The brain lesions in *C. altivelis* are not vacuolative and are mild with degeneration of a localised and small number of neurons (Figs. 236 and 242). In contrast, the retinal vacuolative necrosis is more severe with complete loss of the inner and outer nuclear and plexiform layers with necrotic debris floating in the posterior chamber of the eye and retinal detachment (Figs. 237-239). This may explain the relatively mild impact of the disease given a relatively low cummulative mortality rate. The pathogenesis of nodavirus has been studied in Atlantic halibut (*Hippoglossus hippoglossus*), which indicates that the virus can localise in cells of the central nervous system (CNS) of fish that have survived acute disease. Fish with high numbers of nodavirus-positive cells by immunohistochemistry displayed poor growth rates, even though they were free of neurological symptoms. This occurred over a period of 7 months (Johansen *et al.,* 2004). Thus it would seem that the type of clinical signs from nodavirus is determined by the location and number of neurons infected with the virus in the CNS and this will vary with fish species. Recent molecular immunological studies suggest that the immunogenic viral coat protein 32.5 kDa of VNN in *C. altivelis* can evoke the proliferation of CD-4 and CD-8 glycoprotein expressing immune cells of the brain, heart and kidney that may suppress the proliferation of VNN systemically (Yanuhar, 2011). A potential explanation of why some fish species are more resistant to nodavirus pathology is the ability to produce interferon, which inhibits viral replication. In a study of *Epinephelus coioides*, the family of interferon-inducible genes (*OsgMx)* in all tissues of nodavirus naturally infected grouper was upregulated. Transcription of *OsgMx* gene increased 6 hours after intramuscular injection of nodavirus and this peaked in the fish brains at 72 hours. In healthy control grouper, high levels of OsgMx expression occurred in the eyes, gills and heart compared to post-infection fish which had high levels in many more tissues including the eyes, gills, brain, heart, liver, kidney and intestine. It is possible that grouper *Mx* plays an important role in host defence mechanisms against nodavirus (Chen *et al.*, 2006).

Diagnosis

Diagnosis of VER is based on:

- Demonstration of vacuolative and degenerative changes in the brain and retinal structures.
- Positive PCR result using the primers for RGNNV or Striped Jack Nervous Necrosis Virus (SJNNV) genotype.
- Virus isolation using seabass (SB) (Fig. 245) and striped snakehead (SSN-1) cell lines.
- Positive immunoperoxidase result using SJNNV antibody (Figs. 240- 241 and 243-244).

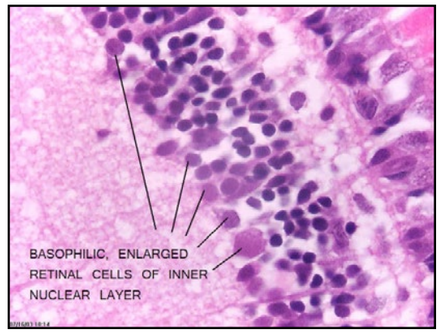


Figure 236. Early changes in retinal cells of basophilia and enlargement; humpback grouper (*Cromileptes altivelis*) with VER.

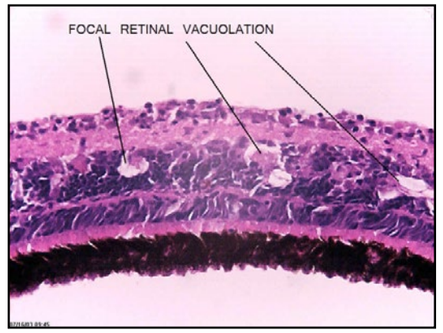


Figure 237. Focal retinal vacuolation; note the remnants of the retinal cell body in the vacuole; Humpback grouper (*Cromileptes altivelis*).

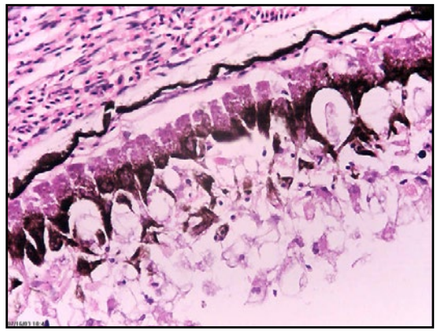


Figure 238. Advanced retinal vacuolation, degeneration and dehiscence of the retinal nuclear layers extending to the pigment cell layer; humpback grouper (*Cromileptes altivelis*) with VER.

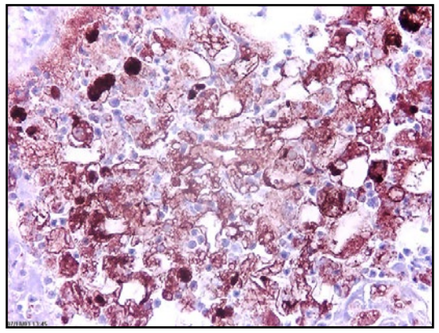


Figure 241. Immunoperoxidase with SJNNV antibody; positive reaction in a necrotic area of vacuolation in the retinal; note intense staining of cell bodies with moderate staining of the rims of the vacuoles; humpback grouper (*Cromileptes altivelis*) with VER.

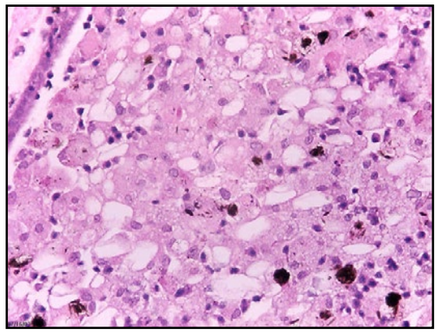


Figure 239. Necrotising retinitis with vacuolation and necrotic cell debris; humpback grouper (*Cromileptes altivelis*) with VER.

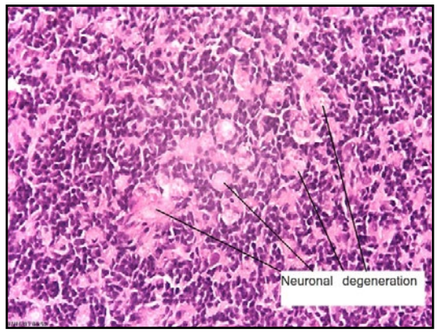


Figure 242. Brain area with neuronal degeneration; vacuolation not developed yet; humpback grouper (*Cromileptes altivelis*) with VER.

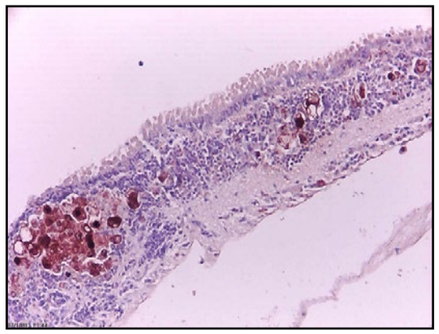


Figure 240. Immunoperoxidase with SJNNV antibody; positive reaction in the focal area of vacuolation in the retina; humpback grouper (*Cromileptes altivelis*) with VER.

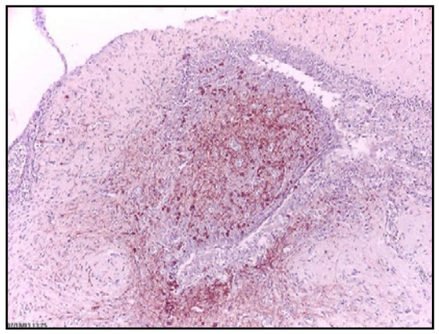


Figure 243. Immunoperoxidase with SJNNV antibody; positive reaction in an area of the brain ; humpback grouper (*Cromileptes altivelis*) with VER.

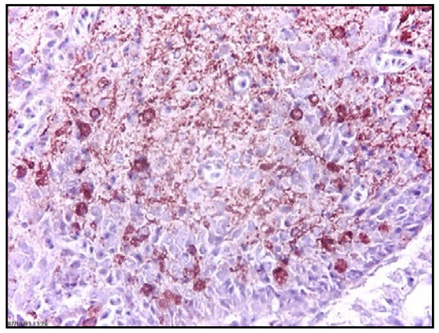


Figure 244. Immunoperoxidase with SJNNV antibody; positive reaction in an area of the brain; individual neurons with intense signal indicating higher viral load; humpback grouper (*Cromileptes altivelis*) with VER.

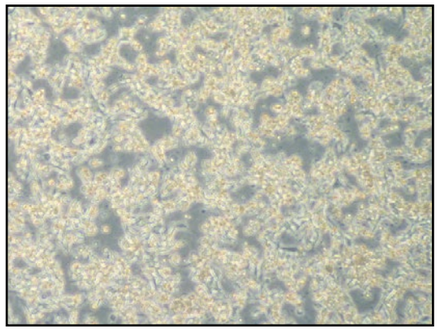


Figure 245. Seabass cell-line showing cytopathic effects (rounding cells and deficits in the cell layer); inoculated with tissue from humpback grouper (*Cromileptes altivelis*) with VER.

The observation of SJNNV antibody produced a positive result while the SJNNV PCR primer did not suggest that the SJNNV antibody is able to cross react with antigens from different nodavirus genotypes.

Control and Prevention

Control measures for VER in marine fingerlings and fry are primarily aimed at:

- Early detection of infection; this would require PCR testing of fish prior to importation with attendant health certification from the exporting authority.
- Quarantine conditions of new batches of fish requiring separation of holding systems; this means either an all-in-all-out with disinfection between batches or separated rooms and biofiltration systems for different batches.
- VER is usually not an issue on grow-out farms, as the virus tends to affect mainly the young life stages of susceptible fish. However, if fish from grow-out farms or the wild are to be sourced for broodstock use, these fish need to be tested using blood or gamete samples for VER PCR.
- Infected fish should be culled from the hatchery system and disinfection with chlorination is required.
- Biosecurity in terms of routine disinfection via chlorinated footbaths, ethanol hand sprays, batch dedicated utensils and hatchery staff training are required to maintain freedom from re-introduction and spread of nodavirus.
- Experimental vaccination of fish has been conducted in Japan where a formalin-inactivated red spotted grouper nervous necrosis virus (RGNNV) was injected once intraperitoneally into juvenile seven banded grouper *Epinephelus septemfasciatus*. Virus-neutralising antibodies developed in vaccinated fish from day 10-160 post-vaccination with higher mean antibody titres from day 21-77. Fish were challenged with homologous virus at 14, 35 and 75 days post-vaccination resulting in lower mortalities in the vaccinated fish compared to controls at a relative percent survival of 67 and higher. A field trial conducted with vaccinated fish challenged by natural infection in net pens resulted in higher survival rates in vaccinated fish at a RPS of 85. This work suggests the potential for the use of an inactivated virus vaccine against nodavirus disease (Nakai *et al.*, 2005).

Grouper Iridoviral Disease

Farm History

Grouper iridoviral disease (GID) is a significant cause of production loss primarily in the grow-out of giant grouper (*Epinephelus lanceolatus*). Mortalities can occur sporadically in green grouper (*Epinephelus coioides*) and brown spotted grouper (*Epinephelus areolatus*). Infection of high finned grouper (*Cromileptes altivelis*) is possible. Losses of seacaged giant grouper occur in the first few days of stocking but onset can be delayed up to 2 months post-stocking. GID affects both smaller and larger (up to 20 cm in total length) *E. lanceolatus* (Figs. 246-248).

Figure 246. Seacaged farming of giant grouper; note close proximity of seacages.

Figure 247. Small numbers of dead fish found per day during the die-off, typical of giant grouper (*E.lanceolatus*) with GID.

Figure 248. Note dead fish with no obvious body lesions and widely opened mouths; giant grouper (*Epinephelus lanceolatus*) with GID.

Clinical Signs

Infected fish become weak, floating near the surface, darkened and die with wide open mouths with few external lesions. The heart has petechial haemorrhages on the ventricle with blood-tinged fluid in the pericardial sac. The swim bladder and spleen can be enlarged. Splenic necrosis and congestion are observed. Pale gills indicating anaemia or gill haemorrhage may be present. The liver may be pale and have patchy congestion (Figs. 249-255).

Figure 249. Giant grouper (*Epinephelus lanceolatus*) mid-cycle fish with GID; note the absence of external lesions.

Figure 250. Green grouper (*Epinephelus coioides*) with GID; except for the pale pigmentation of the whole fish, there are no external lesions.

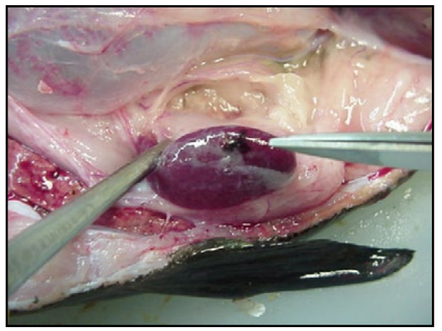


Figure 251. Splenic congestion and enlargement; giant grouper (*Epinephelus lanceolatus*) with GID.

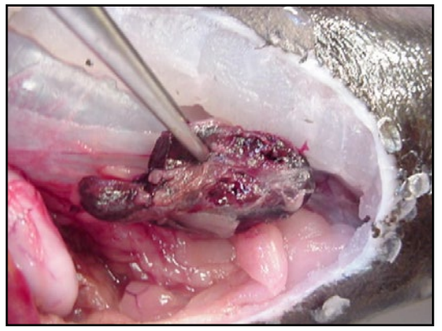


Figure 252. Splenic necrosis; giant grouper (*Epinephelus lanceolatus*) with GID.

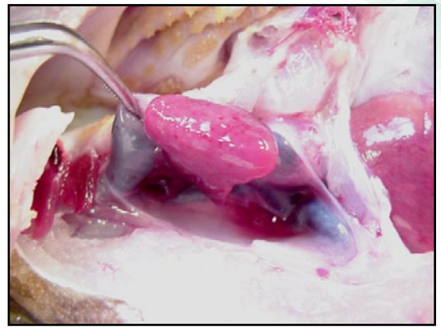


Figure 253. Cardiac ventricular petechiation; giant grouper (*Epinephelus lanceolatus*) with GID.

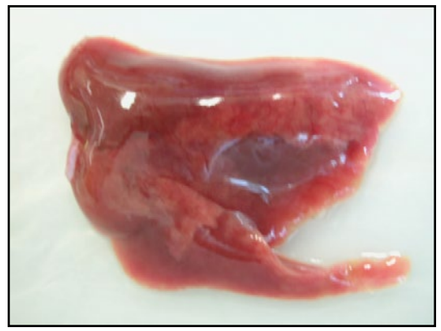


Figure 254. Liver with patchy congestion; giant grouper (*Epinephelus lanceolatus*) with GID.

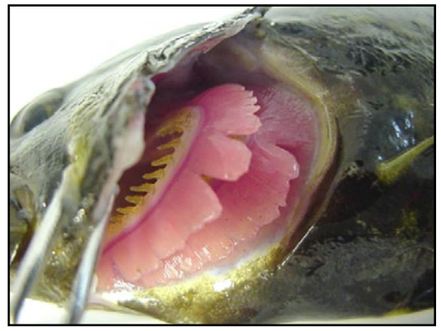


Figure 255. Pale, anaemic gills; giant grouper (*Epinephelus lanceolatus*) with GID.

Epidemiology

The reported mean mortality rate of giant grouper (*Epienephelus lanceolatus*) was 48%,n=8, ranging from 20-92% over a period of 3-4 weeks. In one case of recirculation culture of green grouper (*Epinephelus coioides*), the reported mortality rate was 99% and in one mariculature site the estimated mortality was 26%. Mortality in brown spotted grouper (*Epinephelus areolatus*) was reported at 50-80% from one farm.
Importation of grouper iridovirus (GIV) infected fish appears to be the route of introduction of GID into marine cage culture in Hong Kong. As fish are generally asymptomatic, it is not possible to differentiate healthy fish from infected fish usually until a disease outbreak is occurring on the farm. Disease outbreak is possibly associated with post-transport stress or following changes to local weather, e.g., heavy rain. The focus of GID appears to be in *Epinephelus lanceolatus* with some cross-infection of *E. lanceolatus* and *E. coioides* that may be co-cultured on the farm. There is little evidence of GID affecting non-grouper species such as snapper, cobia or seabream from targeted surveillance data.

Pathophysiology

GIV causes necrosis of the haematopoietic tissues, the liver, heart, kidney and also gill hyperplasia. Infection is associated with the presence of hypertrophied basophilic cells (HBCs) (Figs. 256-263) containing cytoplasmic virions (Fig. 268). These cells appear to resemble infected macrophages. Mortalities may result from extensive haematopoeitic tissues causing clinical anaemia and the stress of intensive culture, e.g., reduced dissolved oxygen levels may result in high cumulative mortalities. Studies on the viral coat protein of Singapore grouper iridovirus (SGIV) suggest that the pathogenesis of the disease is independent of a VP88 protein which is involved in binding to the fish host cell 94 kDa membrane protein (Zhou *et al.*, 2011). The hypertrophic cells in GIV are likely of monocyte origin as they possess phagocytic activity but this was impaired in virus infected cells between 2-4 days post-infection. After infection, the enlarged cells first appeared in the spleen with an abundance peak at 64 hours post-infection and then in the head kidney, trunk kidney and gills in a second peak 120 hours post-infection. Ultra-structurally, heterochromatins of the infected cells are marginated or aggregated to one side of the nuclei during the early stages of the infection. Damage and rupture of the nuclear membrane begins before the formation of the viral particles (Chao *et al.*, 2004).

Diagnosis

Diagnosis of GID is based on the following criteria:

- Gross pathology in the spleen, kidney, liver and gills (Figs. 251-255).
- Giemsa stained smears of kidney, spleen or gills demonstrating the presence of HBCs (Figs. 256-258).
- Histopathology – presence of HBCs in necrotic areas of spleen and kidney. HBCs also present in the gills, heart, liver, thymus and pancreas (Figs. 259-263).
- PCR test using Red Seabream iridovirus (RSIV) primers (Fig. 267).
- IHCT – immunohistochemistry test using RSIV antibodies (Figs. 264-266).
- Transmission Electron Microscopy to demonstrate iridovirus of size 121-143 nm, with icosahedral morphology (Fig. 268).
- Virus culture in Epithelioma Papulosum Cyprini (EPC) and grouper fin (GF). Other cell lines susceptible to grouper iridovirus are grouper kidney (GK-2) and brain cell lines (Yeh *et al.*, 2008).

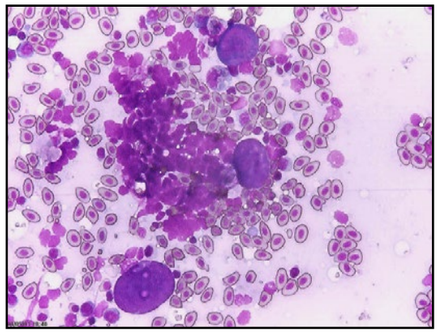


Figure 256. Giemsa stained kidney smear with a macrophage undergoing hypertrophy; note the increasing basophilic staining of the cytoplasm; giant grouper (*Epinephelus lanceolatus*) with GID.

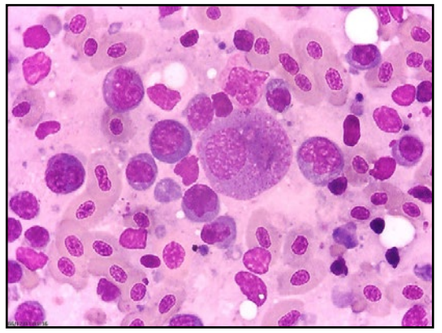


Figure 257. Giemsa stained kidney smear with hypertrophic basophilic cells; HBCs are much larger than normal organ cells with 15 – 22 μm diameters (2-3 times the size of a red blood cell); giant grouper (*Epinephelus lanceolatus*) with GID.

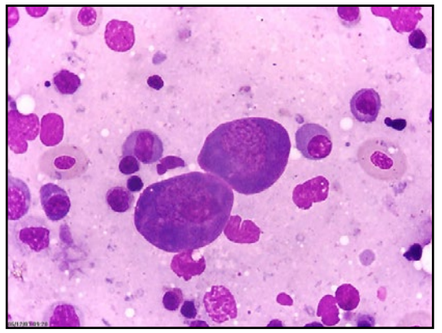


Figure 258. Giemsa stained spleen smear with hypertrophic basophilic cells; giant grouper (*Epinephelus lanceolatus*) with GID.

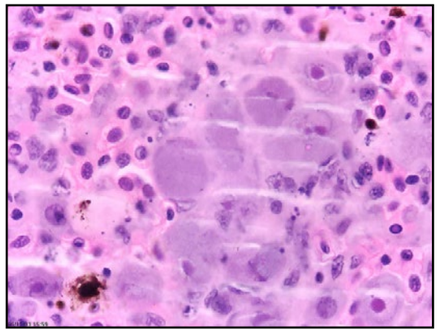


Figure 259. (H&E) Spleen with HBCs; giant grouper (*Epinephelus lanceolatus*) with GID.

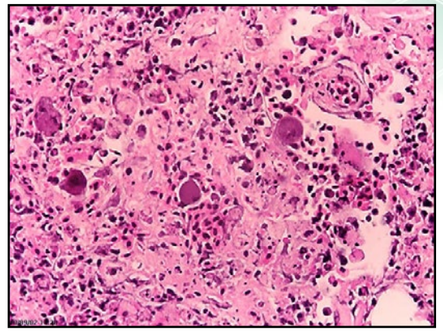


Figure 260. (H&E) Spleen with extensive necrosis and scattered HBCs; giant grouper (*Epinephelus lanceolatus*) with GID.

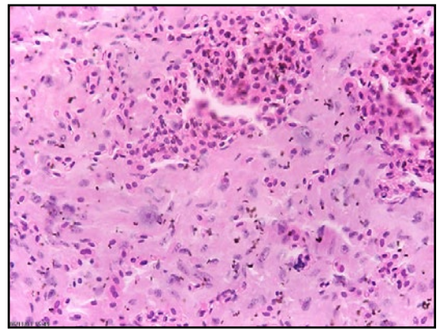


Figure 261. (H&E) Myocardial degeneration and scattered HBCs; giant grouper (*Epinephelus lanceolatus*) with GID.

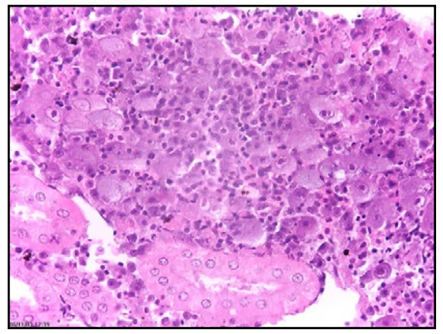


Figure 262. (H&E) Renal haematopoeitic necrosis and large number of HBCs; giant grouper (*Epinephelus lanceolatus*) with GID.

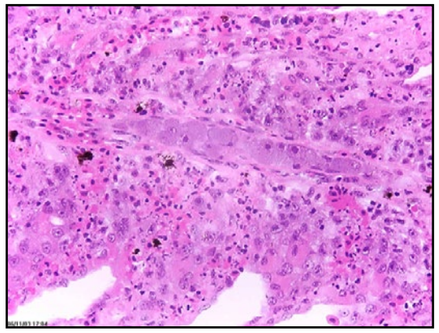


Figure 263. (H&E) Gill hyperplasia with lamellar necrosis and many HBCs; giant grouper (*Epinephelus lanceolatus*) with GID.

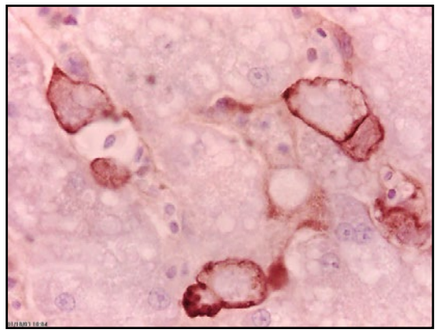


Figure 264. RSIV immunoperoxidase, positive result on spleen hypertrophic cells; giant grouper (*Epinephelus lanceolatus*) with GID.

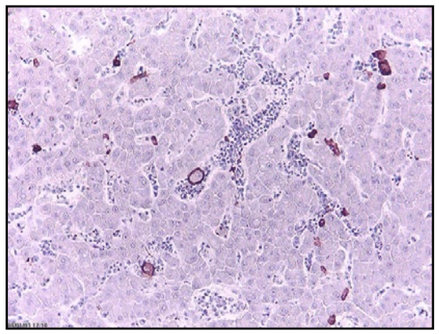


Figure 265. RSIV immunoperoxidase, positive result on gill hypertrophic cells; giant grouper (*Epinephelus lanceolatus*) with GID.

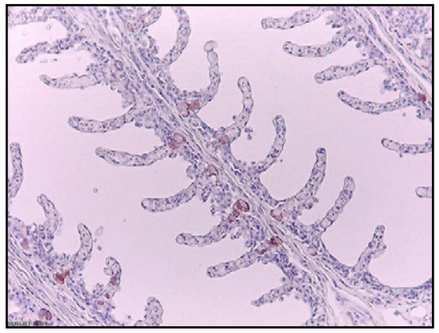


Figure 266. RSIV immunoperoxidase, positive result on liver hypertrophic cells; giant grouper (*Epinephelus lanceolatus*) with GID.

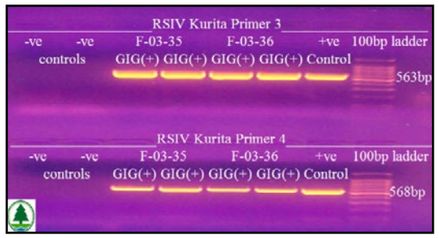


Figure 267. Positive PCR results for RSIV using Kurita 3 and 4 primers on agarose gel; giant grouper (*Epinephelus lanceolatus*) with GID.

While the RSIV primer-based PCR appears to detect grouper species infected with GIV, the PCR product would need to be sequenced to confirm that it is a grouper specific iridovirus.

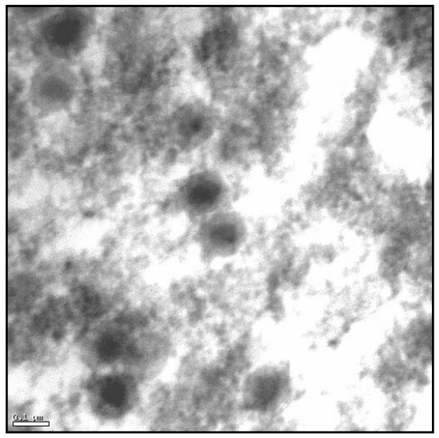


Figure 268. Iridovirus virions of diameter 121-143 nm, icosahedral; giant grouper (*Epinephelus lanceolatus*) with GID; scale bar = 0.1 µm.

Control and Prevention

GID is an example of imported viral diseases. Control strategies would need to be directed at the following:

- Import health testing to distinguish carrier fish from GIV free fish; this would require the use of rapid diagnostic tests such as PCR.
- In a marine farm with infected fish, these should be culled if there does not appear to be widespread dissemination of the virus to co-cultured species. This would limit the spread of the virus to subsequent batches of giant, green or brown spotted groupers.
- The scope of prophylactic vaccination of grouper species against GIV should be explored. Research into Singapore grouper iridovirus using bioinformatics and DNA vaccination based on plasmid DNA encoding ORF072, ORF039 and ORF036 produced 66.7%, 66.7% and 58.3% relative survival rates respectively in comparison to control fish (Ou-Yang *et al.*, 2012).

Seabream Iridoviral Disease

Farm History

Culture of seabream (*Pragus* spp.) in Hong Kong is not a major production activity and is reliant on mainly imported fish fingerlings with some locally caught fish stocks. However, seabream is commonly co-cultured with other marine species. Seabream Iridoviral Disease (SBID) has been detected in imported seabream but not in locally caught seabream species (Figs. 269-270). Farmers reported sustained mortalities that were unresponsive to freshwater bathing employed routinely to treat fish in the first few weeks of arrival.

Figure 269. Juvenile seabream (*Pagrus* sp.) imported for local culture.

Figure 270. Locally caught seabream (*Pagrus* sp.) for cage culture.

Clinical Signs

Gross pathology consistent with but not pathognomonic with SBID includes pale gills, liver discolouration with whitish areas and an enlarged, congested spleen (Figs. 281-283).

Figure 271. Seabream (*Pagrus* sp.) with gill pallor and SBID.

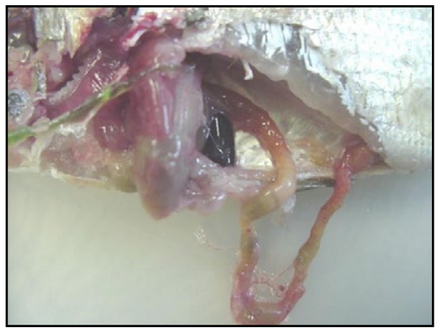


Figure 272. Enlarged congested spleen of seabream (*Pagrus* sp.) with SBID.

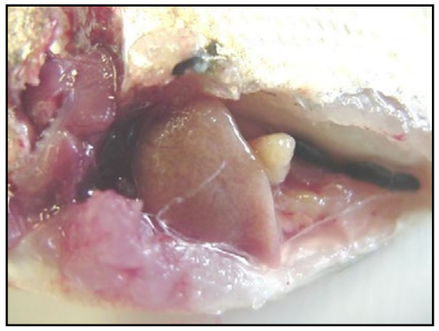


Figure 273. Pale liver with whitish areas; seabream (*Pagrus* sp.) with SBID.

Epidemiology

Seabream Iridoviral Disease (SBID) has only been detected in imported seabream. In terms of understanding the disease caused by the megalocytivirus group of Iridoviridae, it appears that seabream iridoviral disease in Hong Kong is limited to seabream while the grouper iridovirus is host specific for grouper species. Nevertheless, these iridoviruses are very closely related to the extent that diagnostic tests for RSIV can be used to detect GIV. Infected fish may be co-infected with *Photobacterium damsela* and the cumulative mortality reported was 80%.

Pathophysiology

The histopathology of SBID is similar to that observed in GID. There is systemic distribution of hypertrophic basophilic cells (HBCs) in the spleen, kidney, gills, liver and heart. Cellular necrosis is evident primarily in the spleen haematopoietic tissues. Some necrosis occurs in renal melanomacrophage centres (MMCs). Multifocal hepatocellular degeneration and necrosis occur in the liver. There are also gill lamellar changes consisting of hyperplasia, fusion with intralamellar haemolysis, and lymphocytic branchitis (Figs. 274-279).

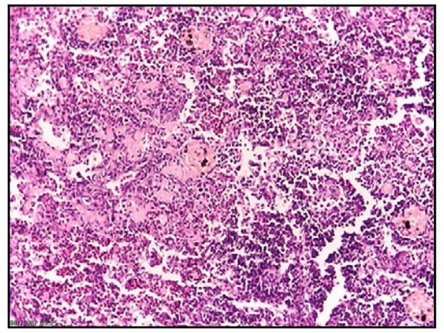


Figure 274. Splenic necrosis; seabream (*Pagrus* sp.) with SBID.

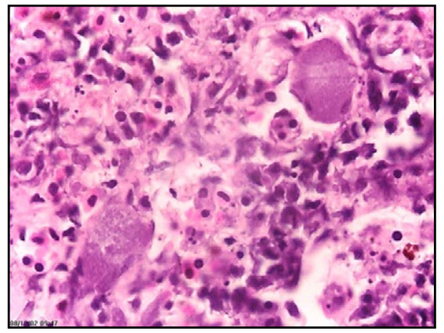


Figure 275. Hypertrophic basophilic cells (HBCs) in the necrotic splenic pulp; seabream (*Pagrus* sp.) with SBID.

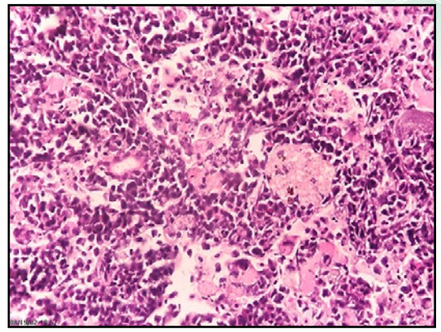


Figure 276. Kidney with focal necrosis in the MMCs; seabream (*Pagrus* sp.) with SBID.

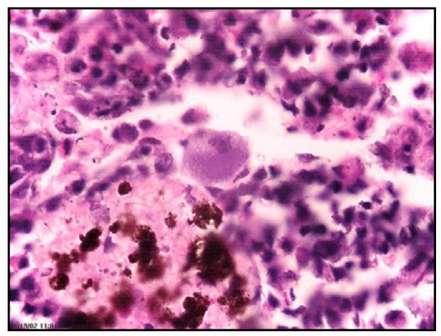


Figure 277. HBCs adjacent to the necrotising MMC of kidney; seabream (*Pagrus* sp.) with SBID.

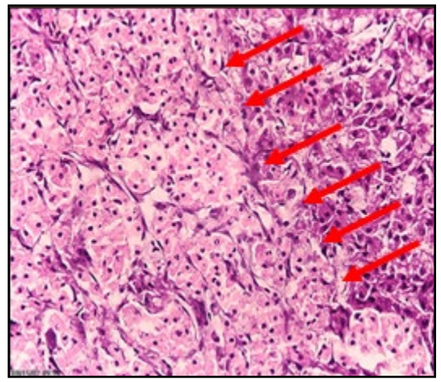


Figure 278. Liver hepatocellular degeneration and necrosis (arrows); seabream (*Pagrus* sp.) with SBID.

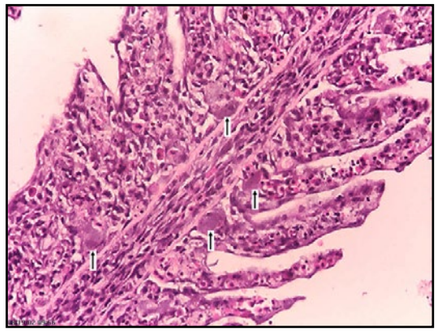


Figure 279. Gills with HBCs (arrows), branchitis, hyperplasia, fusion and haemolysis in the lamellar capillaries; seabream (*Pagrus* sp.) with SBID.

Diagnosis

Diagnosis is primarily based on the presence of HBCs systemically and associated necroses in the spleen, and kidney haematopoietic tissues.

Control and Prevention

Control strategies would need to be directed at the following:

- Import health testing to distinguish carrier fish from SBIV free fish; this would require the use of rapid diagnostic tests such as PCR.
- Infected fish in a marine farm should be culled if there does not appear to be widespread dissemination of the virus to co-cultured species. This would limit the spread of the virus to subsequent batches of imported seabream.
- The scope of prophylactic vaccination of seabream species against SBIV should be explored.

Lymphocystis Disease

Farm History

Lymphocystis is a viral disease observed in marine fish culture and is caused by an iridovirus. Species affected include cobia (*Rachycentron canadum*), giant grouper (*Epinephelus lanceolatus*), tiger grouper (*E. fuscoguttatus*), green grouper (*E. coioides*) and banded head snapper (*Lutjanus* sp.).

Clinical Signs

The clinical signs of lymphocystis are typically the formation of gross nodules of varying sizes on the fins and tail edges. The damaged fins can become swollen, eroded and erythematous. Secondary infection of the infected appendages may occur. There are no internal lymphocystis lesions (Figs. 280-284).

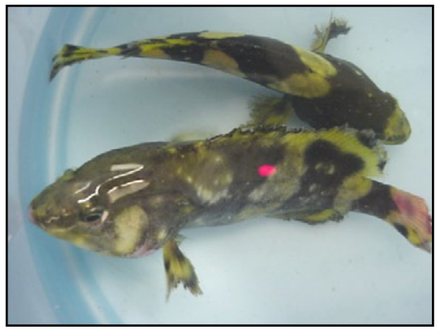


Figure 280. Giant grouper (*Epinephelus lanceolatus*) with frayed fins and blackened nodular edges to the pectoral, dorsal and tail fins; lymphocystis.

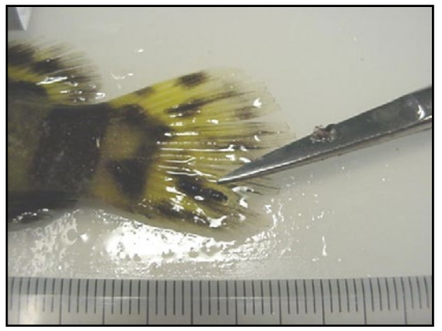


Figure 281. Giant grouper (*Epinephelus lanceolatus*) with blackened nodules on the tail fin.

Figure 282. Banded head snapper (*Lutjanus* sp.) with lymphocystis virus causing black nodules on the tail fin, edges of the dorsal and pelvic fins.

Figure 283. Tiger grouper (*Epinephelus fuscoguttatus*) with nodular tail lesions of lymphocystis.

Figure 284. Cobia (*Rachycentron canadum*) with vegetative (cauliflower-like formations) fin lesions caused by lymphocystis.

Epidemiology

Lymphocystis infections occur mainly in juvenile fish that may have been subject to the stressors of translocation. It also manifests in larger fish. The condition is not considered to result in direct mortalities except where it coincides with vibriosis or flexibacteriosis.

Pathophysiology

Lymphocystis virus infected cells become hypertrophied. Histologically the epidermal cells are affected and these as a group form nodular lesions. The lymphocystic cells have a clear hyalinised cell 'wall' and a central core of basophilic cytoplasm with a distinctly enlarged nucleus (Figs. 285-287, 291). The inflammatory response consists of either surrounding fibrosis with varying degrees of melanomacrophage-melanin aggregation. These lesions are entirely external.

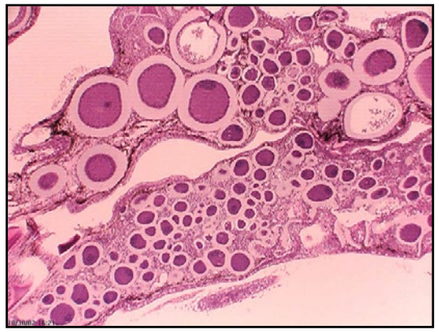


Figure 285. Tiger grouper (*Epinephelus fuscoguttatus*) skin with varied sizes of hypertrophied lymphocystic cells.

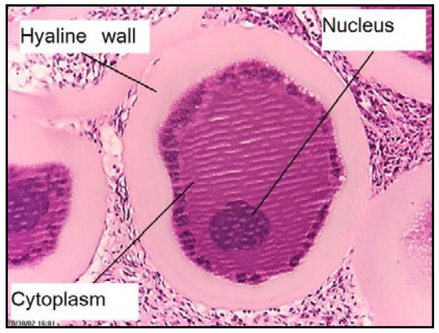


Figure 286. Lymphocystic cell components; tiger grouper (*Epinephelus fuscoguttatus*).

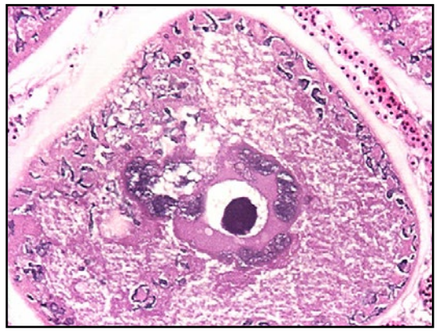


Figure 287. Lymphocystis cell from Cobia (*Rachycentron canadum*).

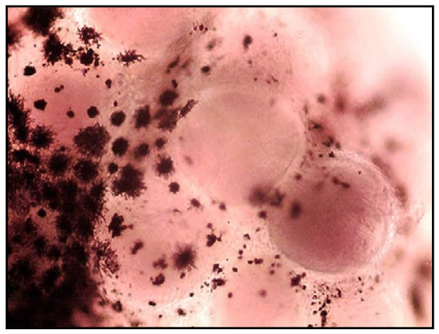


Figure 288. Wet mount of lymphocystis nodules; note the melanophores; tiger grouper (*Epinephelus fuscoguttatus*).

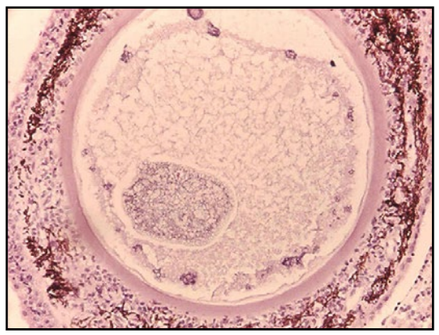


Figure 289. Melanomacrophages and fibrocyte reactions to the lymphocystis cell; tiger grouper (*Epinephelus fuscoguttatus*).

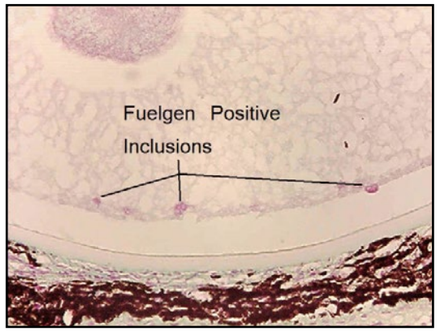


Figure 290. Fuelgen positive inclusions in the cytoplasm of a lymphocytic cell; tiger grouper (*Epinephelus fuscoguttatus*).

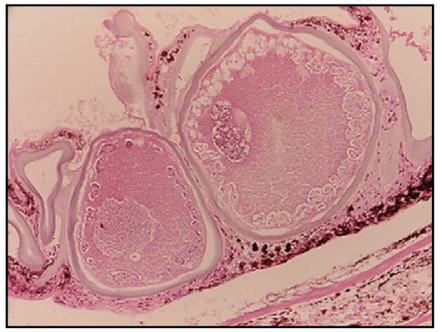


Figure 291. Mature lymphocystis cells; note the halo-effect around the periphery of the cytoplasmic inclusions; green grouper (*Epinephelus coioides*).

Diagnosis

Lymphocytis infection should be differentiated from neoplasia, epitheliocystis and other virally induced dermal growths primarily through histopathological features. A Fuelgen DNA positive stain of the cytoplasm can be indicative of DNA viral infection (Fig. 290). Presumptive diagnosis on a wet squash mount of nodular skin lesions is possible (Fig. 288).

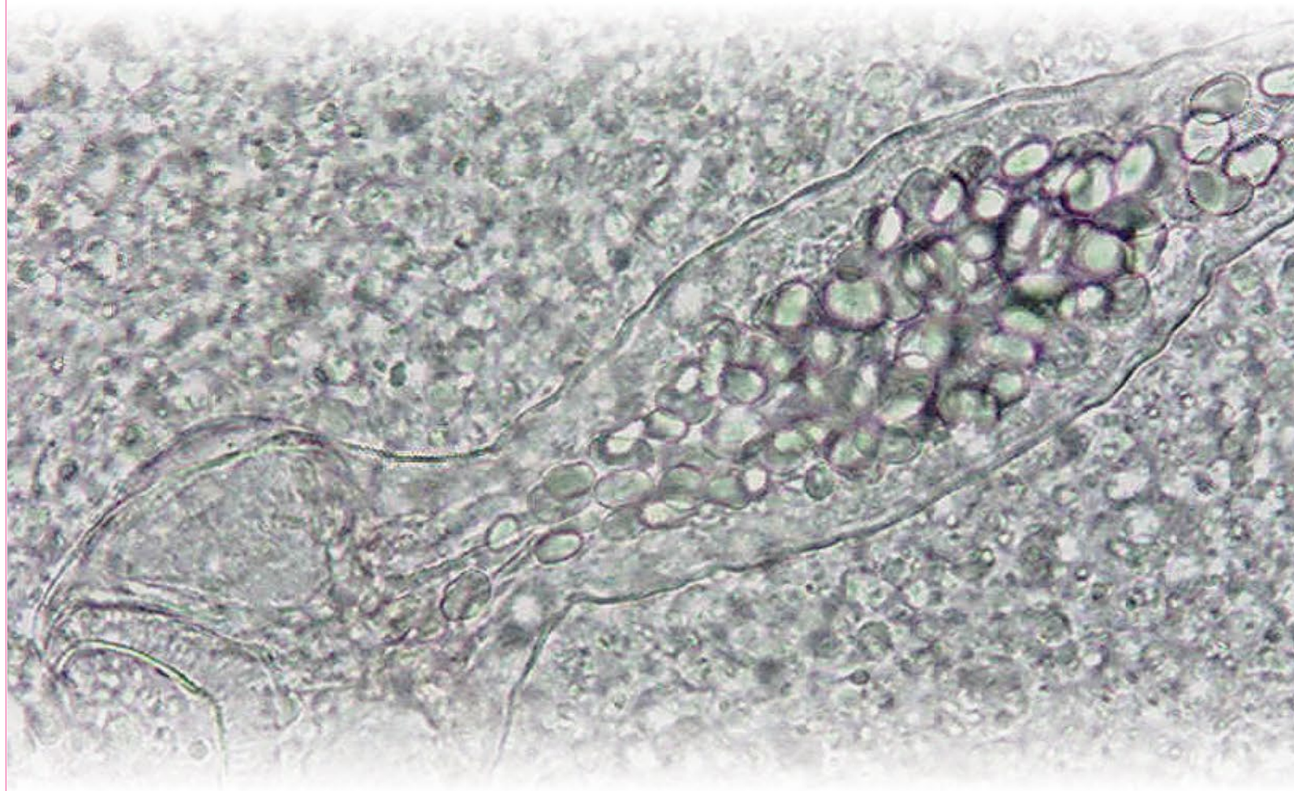
Control and Prevention

In general lymphocystis undergoes self-resolution when the husbandry conditions are improved with respect to:

- Reduction of stocking density, which can alleviate fin nipping and spread of the virus
- Improvement of water quality
- Treatment of secondary bacterial infections

Recent work on recombinant DNA vaccine using lymphocystis as a model suggests that it is feasible to reduce the prevalence of the disease in Japanese flounder. A fragment of the major capsid protein encoding gene from a lymphocystis isolate from Mainland China was cloned into a eukaryote to produce a recombinant plasmid pEGFP-N2-LCDV-cno. 6 kb. When experimentally injected into Japanese flounder intramuscularly and hypodermally, vaccinated fish exhibited a marked reduction in prevalence of lesions for up to 2 months post-challenge (Zheng *et al.*, 2011).

PART 5 – PARASITIC DISEASES



PART 5 – PARASITIC DISEASES

Dactylogyrosis and Gyrodactylosis

Farm History

Gill flukes (*Dactylogyrus* sp.) are very common in locally cultured marine and freshwater cultured fish. Skin flukes (*Gyrodactylus* sp.) occur occasionally. Whether it is an issue for fish health depends on the worm burden and other infections or stress factors such as handling.

Clinical Signs

Gill flukes in heavy numbers of > 400 per fish (e.g., approximately 580 flukes in white blotched snapper of 8 cm in length and approximately 5,500 flukes in *E. lanceolatus* of 24 cm in length) may exhibit increased mucus production, which produces pallor of the gills. If organic sediments are attached to the excessive mucus or where there is secondary bacterial infection, further discolouration of the gills occurs. Fish with severe gill fluke burdens may show rapid or laboured opercular movements, which is a symptom of oxygen insufficiency due to the damaged gills or the poor gas exchange due to the excessive mucus. Skin flukes occur mainly on the skin and may lead to excessive skin mucus. Infected fish may display flashing behaviour in an attempt to alleviate the irritation caused by the flukes (Figs. 292-299).

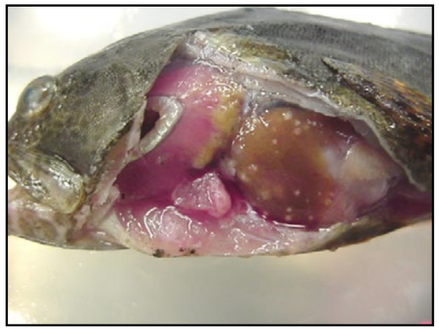


Figure 292. Gill necrosis and pallor associated with gill flukes; an estimate of 660 flukes/fish determined; tiger grouper (*Epinephelus fuscoguttatus*).

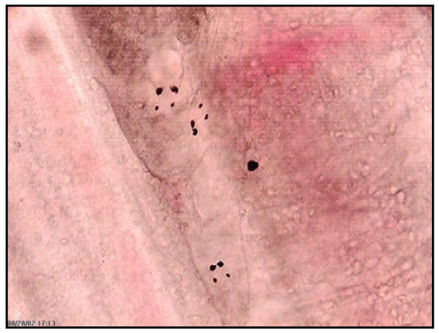


Figure 293. Wet mount of gills showing up to three attached dactylogyrid flukes each with four black eyespots, sandwiched between filaments; tiger grouper (*Epinephelus fuscoguttatus*).

Figure 294. Gill pallor, excessive mucus and necrosis in giant grouper (*Epinephelus lanceolatus*) **with an estimated 5,500 flukes.**

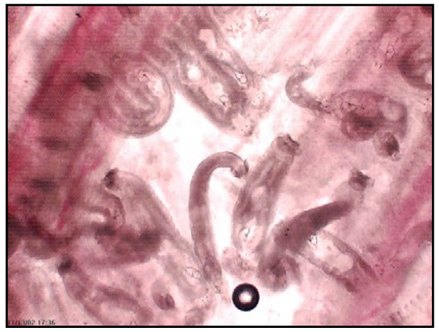


Figure 295. Extremely heavy loading of dactylogyrid flukes; giant grouper *(Epinephelus lanceolatus).*

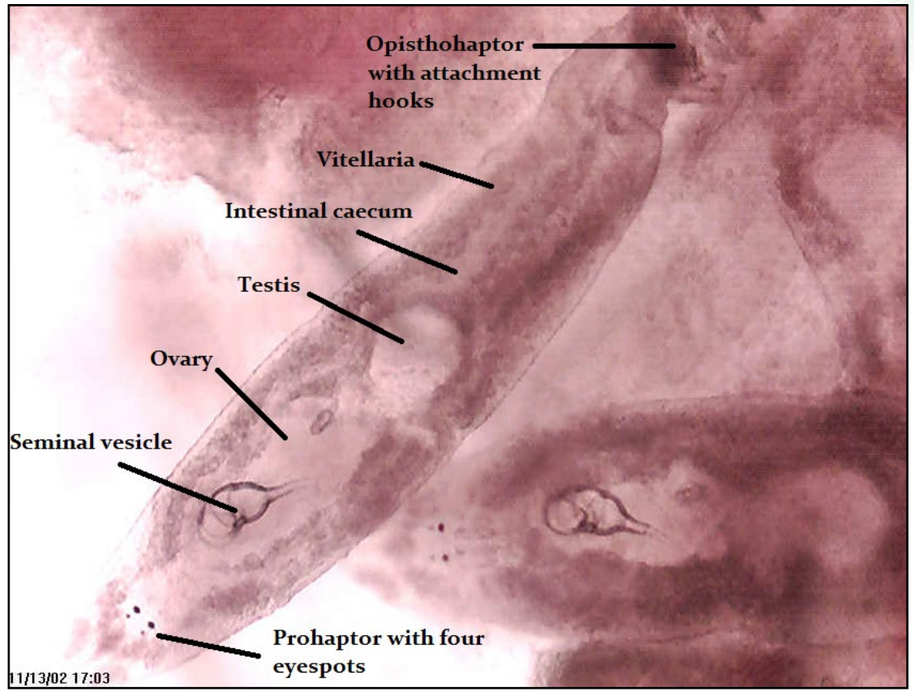


Figure 296. Anatomy of the dactylogyrid fluke; giant grouper *(Epinephelus lanceolatus).*

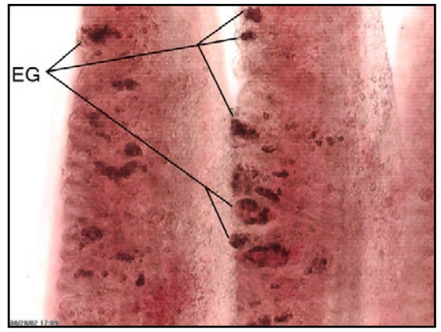


Figure 297. Dactylogyrid fluke eggs (EG) embedded in the gill filaments of tiger grouper (*Epinephelus fuscoguttatus*).

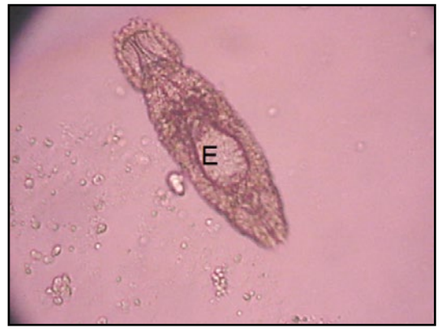


Figure 298. Skin gyrodactylid fluke from goldfish *(Carassius auratus)*; note the lack of eyespots and the location of the developing embryo (E).

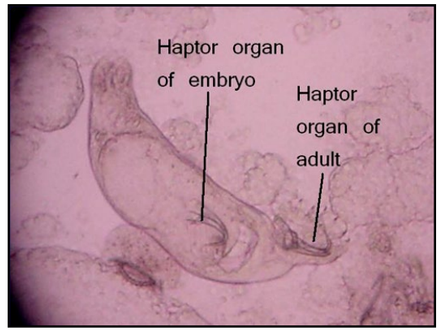


Figure 299. Anatomy of the gyrodactylid fluke *from goldfish (Carassius auratus);* note the excessive mucus around the fluke. There is also a trichodinid adjacent to the fluke.

Epidemiology

Dactylogyrid flukes have a direct life cycle involving the production of eggs. Gyrodactylids are oviparous, producing live young. As a result, direct transmission of the flukes occurs and the greater the stocking density of host fish, the higher the risk of infestation with flukes. In conjunction with high fish density is organic pollution and poor water quality, both of which lead to rapid multiplication of flukes and expression of disease (Reed *et al.*, 2009).

Pathophysiology

Flukes cause damage to the gills and skin primarily through their attachment and feeding activities on the epithelial host surface. The damaged integument may then be subject to secondary bacterial infections. As part of the host response, hyperplasia and mucus production may compromise the capacity of the gills for gaseous exchange. This is due to thickening of the respiratory membrane, which increases the diffusion distance. In heavy infestations, degenerative changes and erosions of the gill and skin may occur. The rubbing of skin by the fish also contributes to integument damage (Figs. 300-302).

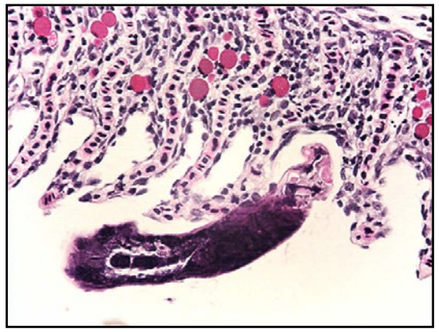


Figure 300. Inter-lamellar hyperplasia and dactylogyrid fluke in discus (*Symphysodon discus*).

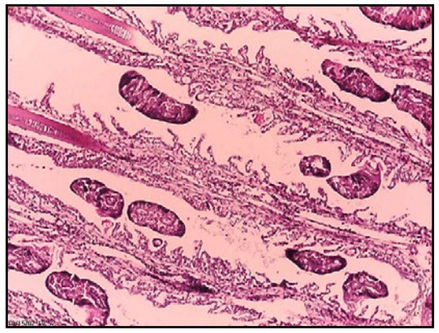


Figure 301. Gill lamellar degeneration due to heavy dactylogyrid fluke infestation in tiger grouper (*Epinephelus fuscoguttatus*).

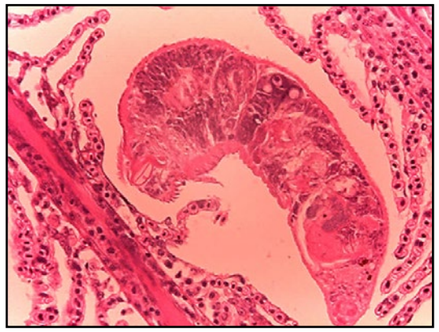


Figure 302. Dactylogyrid fluke causing a compressive lesion at the site of the haptor as well as displacement of adjacent gill lamellae; green grouper (*Epinephelus coioides*).

Diagnosis

Wet mount examinations of the gills and skin readily identify the presence of flukes. To estimate of the numbers of flukes, see the parasitology section for a method assessing parasite loads. Dactylogyrids have four eyespots (Figs. 293 and 296) and produce eggs (Fig. 297). Gyrodactylid flukes do not have eyespots and usually contain a developing embryo within the body (Fig. 299).

Control and Prevention

Heavy infestations can be managed as follows:

- Formalin bathing
- Trichlorphon bathing
- Antibiotic treatment of secondary bacterial infections
- Reduction of stocking density
- Freshwater bathing for marine fish to alleviate stress
- Additional aeration
- Repeated treatments are often necessary

Prevention strategies are aimed at:

- Regular wet mount checks of fish to assess fluke burdens
- Treatment of moderate loads to prevent serious infestations
- Optimising stocking density to avoid crowding and high organic excrement
- Ensuring good water exchange
- Ensuring hygiene of nets and equipment

Benediniasis

Farm History

Benediniasis caused by the capsalid fluke *Benedinia* sp. is seasonally a disease of marine fish culture affecting grouper, snapper and amberjack species, particularly during autumn and winter months.

Clinical Signs

Capsalids are transparent and move over the skin of the fish amongst the excessive mucus produced by the fish. This mucus makes the fish lose its normal shiny, wet appearance to become dull and whitish. Fish may rub on surfaces and create skin and fin ulcerations, depigmentation and erythema. In eye infestations, the cornea becomes eroded and cloudy. Infected fish become inappetent, lose condition and mortalities occur in heavy infestations particularly with secondary vibriosis (Figs. 303-307).

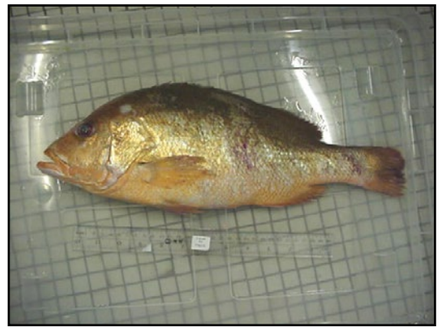


Figure 303. Red snapper (*Lutjanus malabaricus*) with body erosions due to benediniasis; note the loss of shine on the ventro-lateral caudal half of the fish.

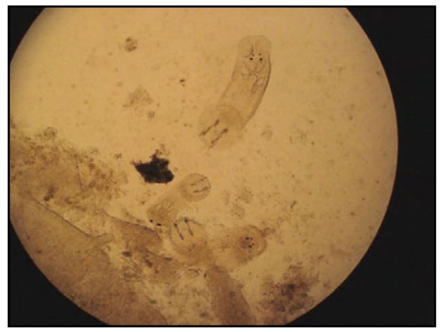


Figure 304. Heavy burden of capsalid flukes from the red snapper (*Lutjanus malabaricus*) in Fig. 303; skin scrape wet mount.

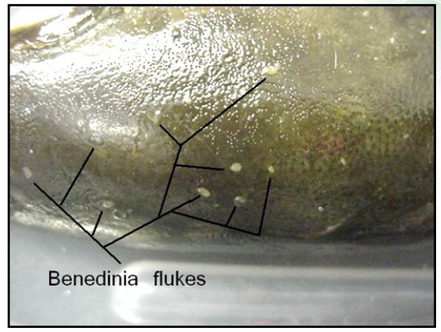


Figure 305. Capsalid flukes become opaque when exposed to freshwater and are dead. Local farmers call the disease ‘white sesame seed’ due to the colour, size (1-2 mm) and shape of the dead flukes. Greater amberjack (*Seriola dumerili*).

Figure 306. Malabar grouper (*Epinephelus malabaricus*) with skin erosions, colour loss and emaciation (big head and thin body) due to capsalid flukes and scuticociliatosis.

Figure 307. Brown spotted grouper (*Epinephelus areolatus*) with heavy benediniasis causing corneal erosion and opacity.

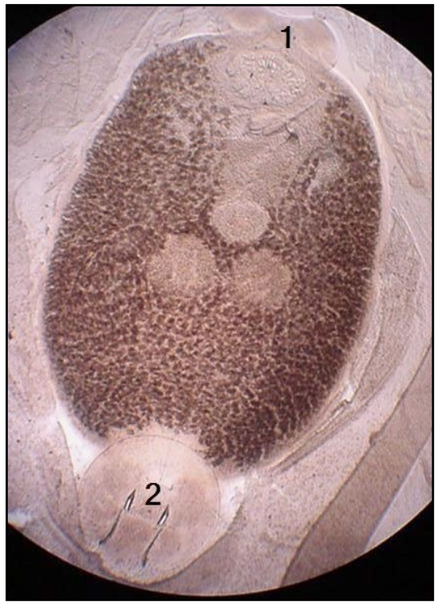


Figure 308. Adult *Benedinia* capsalid fluke; 1 = oral suckers, 2 = haptor organ for host attachment.

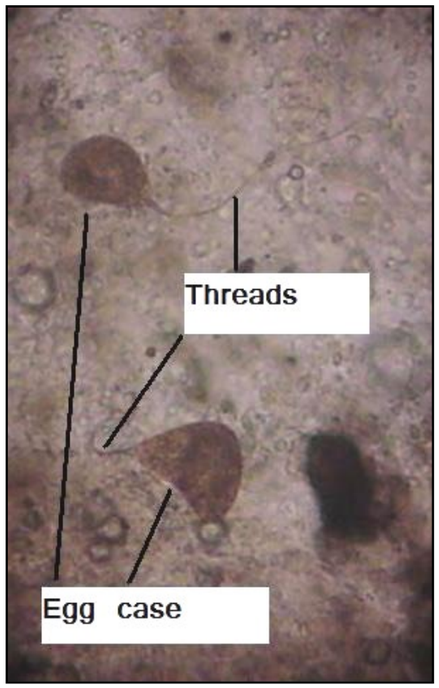


Figure 309. *Benedinia* eggs with triangular egg cases and attachment threads per egg.

Epidemiology

Mortality rates from benediniasis are exacerbated by secondary bacterial infections. Capsalid flukes produce eggs that are diamond or triangular shaped and have a tail-like thread for attachment to fish. In cooler temperatures, the immune response of fish to infestation is probably slower and hence outbreaks are more common. Repeated treatments are required when water temperatures are < 20ºC and fish recover better at > 25ºC.

Pathophysiology

Capsalid flukes typically cause integument and corneal damage through their attachment and feeding activities on the host. The host response is usually increased mucus production and physical rubbing. Damaged integument allows secondary bacterial pathogen invasion.

Diagnosis

Wet mount demonstration of large, flat and ovoid flukes with prominent oral suckers and haptor organ with hooks; eggs of *Benedinia* may be observed (Fig. 309). Application of fresh water to the wet mount kills the flukes and makes them opaque and more visible grossly. Four eyespots near the suckers may be visible (Fig. 308). The capsalid flukes are within the same group as the dactylogyrids and gyrodactylids because of the single unit for the opisthohaptor morphology, hence termed monopisthocotylea. This is distinct from the polyopisthocotylea which have multiple muscular suckers or clamps for the attachment organ (Noga, 2010).

Control and Prevention

If detected early, benediniasis is readily treatable using freshwater bathing for 5 minutes. Once weekly bathing of batches of infected fish is required in the cooler months to control rapid reinfestations. Reduction of stocking density and control of secondary vibriosis or flexibacteriosis is often required in cases of delayed detection. Due to the labour-intensiveness of freshwater bathing, oral dosing with praziquantel may be used as an alternative if cost is not an issue.

Cryptocaryoniasis and *Ichthyophthirius multifiliis*

Farm History

White spot or Ich is an occasional disease in mariculture and ornamental fish trading. It is fairly well recognised by farmers and thus detected and treated early in the course of the infestation before it has a chance to produce significant mortalities. The protozoa have a wide species range with *Cryptocaryon irritans* found in marine species and *Ichthyophthirius multifiliis* in freshwater species.

Clinical Signs

Ich typically expresses disease as white spots of about 1 mm in diameter on the skin and gills. However the absence of these spots does not preclude infestation by the parasite. In fact, most cases of Ich are detected before the formation of white spots. Infected fish often have increased mucus production, which gives them a pale to whitish appearance and pallor on the gills (Fig. 310).

Figure 310. Pallor and mucus on the gills of grouper (*Epinephelus* sp.) with an estimated 87 *Cryptocaryon irritans* trophonts per gill mount.

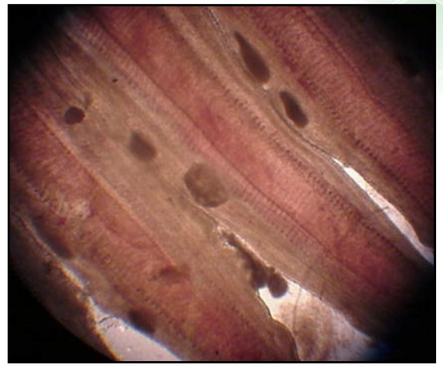


Figure 311. *Cryptocaryon irritans* trophonts in grouper (*Epinephelus* sp.) gill mount; note the flattened shape of the embedded parasites.

Figure 312. *Cryptocaryon irritans* tomont in gill mount of snapper (*Lutjanus* sp.); note the rounded shape of the excysted parasite.

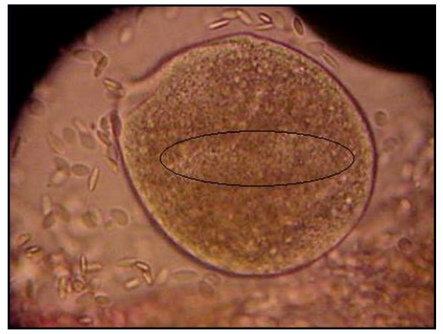


Figure 313. *Cryptocaryon irritans* tomont, which does not have an obvious nucleus; outline of slightly whitish nuclear area provided.

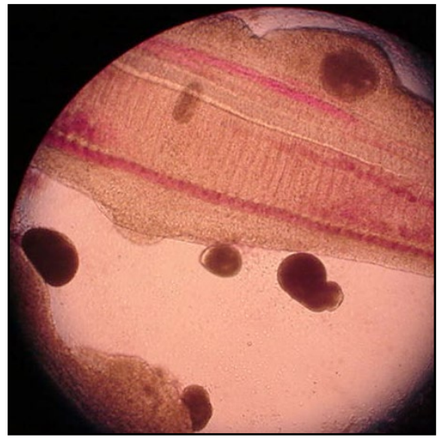


Figure 314. *Cryptocaryon irritans* trophonts in giant grouper (*Epinephelus lanceolatus*) gill mount; note the encysted trophont within hyperplastic gill lamella and a few excysted tomonts.

Epidemiology

Both Ich species have direct life cycles involving encysted trophont, excysted tomonts and free-swimming tomite stages. For *Cryptocaryon irritans* the life cycle may take 11-15 days (Noga, 1996) with optimum replication at 21-24ºC in 6 days. For *Ichthyophthirius multifiliis* at 24-26ºC it takes 3-7 days for the life cycle to complete. In general, the lower the water temperature, the slower the life cycle turnover time.

Pathophysiology

The Ich parasites cause host damage by initiating a hyperplastic response in the infested gills (Fig. 315) and skin. When the trophont stage excysts, the rupture of the gill and skin sites leads to loss of integument integrity and consequent osmotic stress in the skin/gill areas and respiratory compromise in the gills.

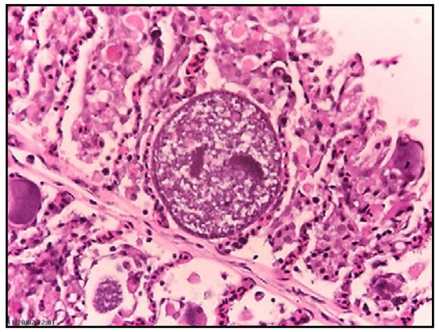


Figure 315. *Cryptocaryon irritans* trophont; note the multilobed nucleus, and gill lamellar hyperplasia around the trophont.

Diagnosis

A wet mount of gill and skin scraping is essential to the detection of the trophont stage of Ich. *Ichthyophthirius multifiliis* trophonts are large cells with a horseshoe shaped nucleus that appears translucent. *Cryptocaryon irritans* trophonts have a hidden nucleus (hence 'cryptic'). Both Ich species show a rotational motion associated with peripheral cilia (Figs. 311-314).

Control and Prevention

Cryptocaryon irritans is amenable to control by repeated hyposalinity or freshwater bathing, which should result in faster resolution at warmer temperatures as the life cycle period is shortened. The excysted tomont stage is susceptible to lysis (Noga, 1996).

Ichthyophthirius multifiliis requires salt bathing to kill the released and free-swimming tomites, as the embedded trophonts are immune to chemical treatment.

Alternative to control is formalin bathing. The key to successful control is early diagnosis and treatment before the rapid multiplication of the parasite into the trophont stages. Once the fish has many white spots, the excysting of the trophonts usually leads to fish mortality even with treatment.

Brooklynellosis and Chilodonellosis

Farm History

Brooklynellosis occurs commonly in marine culture, affecting grouper, snapper and seabream species. Again its impact is heightened by co-infestation with gill flukes, capsalid flukes and *Cryptocaryon irritans,* and secondary bacterial infections. Chilodonellosis is an occasional problem in freshwater fish including ornamental species.

Clinical Signs

In brooklynellosis, infected fish show increased mucus production on the skin and gills. There will be varying degrees of self-trauma with skin and fin abrasions (Fig 318). Secondary infections produce ulceration, erythema and haemorrhage. Gill necrosis is likely in heavy infestations producing white or pale areas of gill rot (Figs 316-319). These fish are lethargic, inappetent and float near the surface.

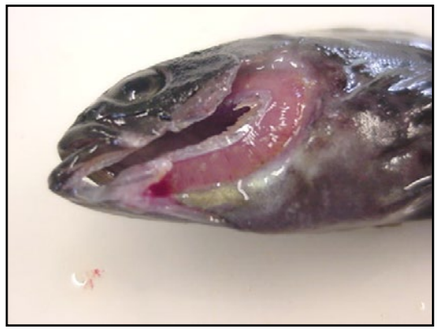


Figure 316. Green grouper (*Epinephelus coioides*) with pale gills that have necrotic white areas; brooklynellosis.

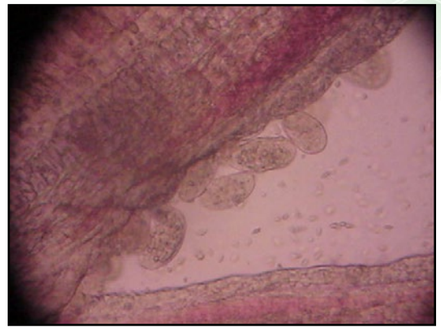


Figure 317. Green grouper (*Epinephelus coioides*) gill wet mount showing numerous *Brooklynella* sp. ciliates feeding on the lamellae.

Figure 318. White blotched snapper (*Lutjanus rivulatus*) with skin abrasions and scale loss in areas around the head and flanks due to brooklynellosis.

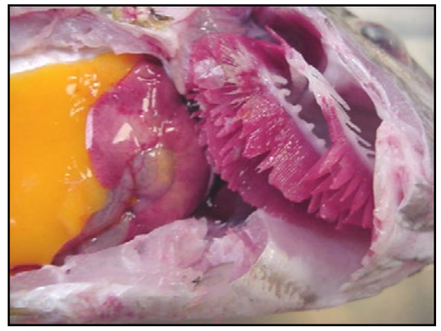


Figure 319. White blotched snapper (*Lutjanus rivulatus*) with gill necrosis (white and ragged area) due to brooklynellosis.

Epidemiology

Brooklynellosis is typically a disease in the cooler months of the year, probably associated with a decline in the immune responsiveness of fish. Overcrowding and increased suspended organics in the water lead to clinical outbreaks. Con-infestation with other parasites and secondary vibriosis is commonly observed in diseased fish (Fig. 321).

Pathophysiology

Hyperplasia of the gill lamellae is a common response to brooklynellosis. This together with increased mucus production and lamellar necrosis (Fig. 320) can result in respiratory and osmoregulatory compromise. The increased mucus also increases the risk of sediment adherence, which further worsens gill function.

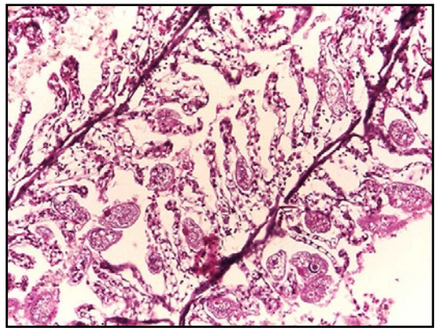


Figure 320. Lamellar necrosis due to heavy infestation of *Brooklynella* sp.

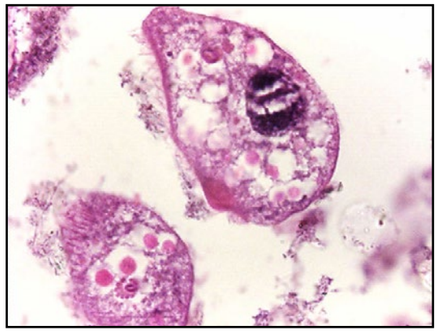


Figure 321. (H&E) *Brooklynella* sp.; note the bacterial clumps associated with the ciliates.

Diagnosis

Wet mount examination of the gills and skin will readily enable detection of *Brooklynella* and *Chilodonella* sp. ciliates. These are ovoid-shaped, have parallel grooves or bands of cilia longitudinally, prominent cytoplasmic vacuoles and a nucleus (Figs. 322-325). Depending on the angle of view, a notch or indentation of the cell membrane may be observed. They usually move with a gliding and rotating motion over the gill, skin or mucus surfaces.

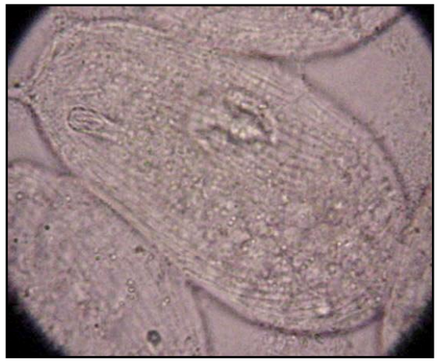


Figure 322. Wet mount of *Brooklynella* sp., top view; note the ciliary bands or grooves.

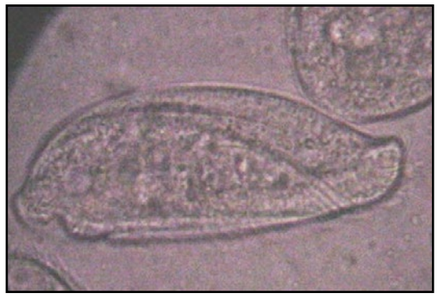


Figure 323. Wet mount of *Brooklynella* sp., side view.

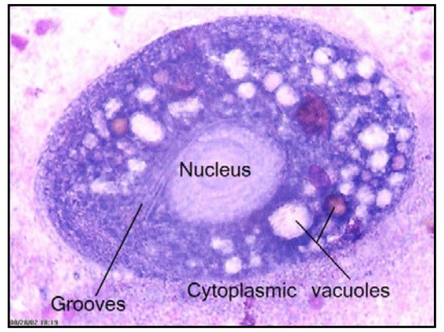


Figure 324. Giemsa stained smear of *Brooklynella* sp.

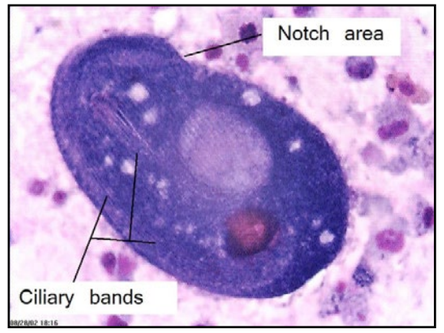


Figure 325. Giemsa stained smear of *Brooklynella* sp.

Control and Prevention

Management of brooklynellosis requires:

- Early detection to avoid secondary bacterial infections, which aggravate mortalities; hence periodic gill and skin wet mounts are important.
- Freshwater bathing of fish to reduce infestation loads with repeat treatments.
- Where bacterial infection is present, a suitable antibiotic treatment is required.
- Infected batches of fish should have reductions in stocking density per cage and supplementary aeration.

Management of chilodonellosis requires:

- The same monitoring checks as for brooklynellosis
- Salt water bathing to reduce infestation loads with repeat treatments
- Management of secondary bacterial infections

Scuticociliatosis

Farm History

Scuticociliatosis disease occurred in *E. malabaricus* grouper, resulting in mortalities of up to 60% in a marine sea cage farm. The fish were also co-infected with *Benedinia* monogeneans and also filamentous Gram-negative bacteria.

Clinical Signs

Infected grouper displayed severe skin erosions and erythema with depigmentation of the skin. Sick fish were very lethargic and floated upside down in the net pen (Figs. 326-327).

Figure 326. Malabar grouper (*Epinephelus malabaricus*) with skin erosions, erythema, very lethargic and floating at the surface due to *Uronema* and capsalid flukes.

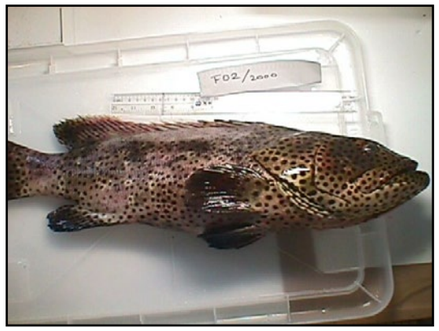


Figure 327. Malabar grouper (*Epinephelus malabaricus*) with skin erosions, erythema, depigmentation due to *Uronema* and capsalid flukes.

Epidemiology

The water temperature was low at 17.3 ºC and the hardness was also low at 117-150 mg $CaCO_3$/L although pH was normal at 8.5. Scuticociliatosis affects tropical marine aquarium species but recently has also been a problem in Japanese flounder, turbot and European seabass culture in the Mediterranean (Noga, 2010). *Uronema marinum* has a wide host range and can infect fish over a wide temperature range of 8-28 ºC and salinity of 20-31 ppt (Cheung *et al*., 1980).

Pathophysiology

The protozoan ciliate *Uronema marinum* has the ability to burrow deep into the skin and internal organs of the host resulting in systemic disease. It is the marine counterpart of *Tetrahymena*, the cause of guppy killer disease, which is an acute condition with fish appearing normal one day and dead the next. *U. marinum* of Atlantic and pacific marine fish result in heavy infections of gills, viscera and body muscle, the cause of death being the gill damage (Cheung *et al*., 1980). In this case the *Uronema* sp. infection was localised to the skin (dermis) (Fig. 328) and a number of parasites were found on gill scrape examination. There was no apparent systemic infection into the internal viscera. However the depigmentation of the skin over the flank areas and the large number of organisms at the dermal layer suggested that deep invasion into muscle was possible.

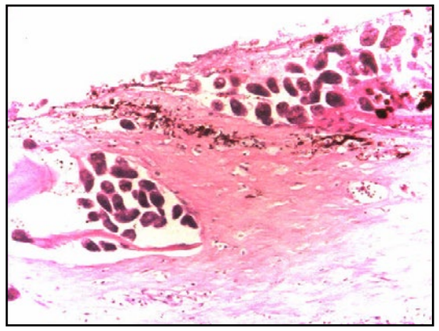


Figure 328. (H&E) Skin of Malabar grouper (*Epinephelus malabaricus*) showing aggregations of *Uronema*-like parasites making their way deeper through the dermis; note the eroded overlying epidermis.

Diagnosis

Scuticociliatosis can be readily diagnosed by skin and gill wet mount examinations, looking for small, ellipsoid, free swimming ciliates (Fig. 329) with cytoplasmic vacuoles, which are similar to but smaller than Brooklynella ciliates. They also need to be distinguished from *Cryptocaryon irritans*, which are larger and have a macronucleus. Definitive speciation requires examination of the cilia pattern on the *Uronema*-like ciliate and PCR testing. Scuticociliatosis can also be caused by *Phiasterides dicentrarchi, Psudocohnilembus persalinus* (Kim *et al*., 2004a, 2004b) or *Miamiensis avidus* (Jung *et al*., 2007).

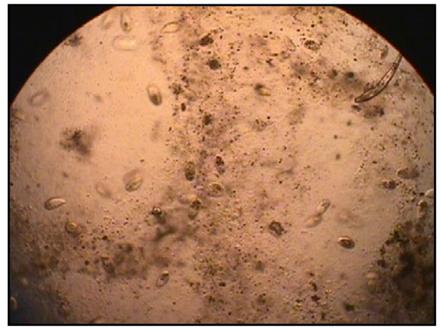


Figure 329. Skin wet mount scraping showing the number of ellipsoid ciliates that are *Uronema*-like in morphology.

Control and Prevention

Once the infection becomes systemic, treatment is usually ineffective with formalin or freshwater bathing. The latter treatments need to be applied very early in the course of the disease. Systemic infections have been treated with metronidazole for *Tetrahymena* in golden perch (*Macquaria ambigua*) (Herbert, 2005). In vitro assays with *Philasterides diicentrarchi* indicated in decreasing order of potency that niclosamide, oxyclozanide, bithionol sulfoxide, toltrazuril, 2-chloro-4-nitroaniline, furaltadone, doxycycline hyclate, formalin, albendazole, carnidazole, pyrimethamine, quinacrine hydrochloride and quinine sulfate have some potency to kill the ciliates within a 24h assay (Iglesias *et al*., 2002). It must be noted that none except formalin are registered agents for use in fish, so more research and validation studies are required for safety and efficacy in field situations.

Nematodiasis

Farm History

Round worms occasionally occur in fish and are of concern to food safety regulators. Due to the open culture system of mariculture, exposure to wild fish species can have an influence on the incidence of nematodiasis and other helminth infestations in fish. In ornamental fish, nematodiasis also occurs but is rarely associated with disease outbreaks.

Clinical Signs

Nematodiasis can lead to loss of body condition and anaemia in severe infestations. This however is an uncommon finding in both the mariculture and ornamental fish sectors in Hong Kong. Usually nematodes are found on routine necropsy examination of the viscera with relatively low numbers of worms either at adult or larval stages in the gastrointestinal tract. Occasionally wild caught fish, e.g., rabbit fish may have heavy nematode burdens (Figs. 330-336).

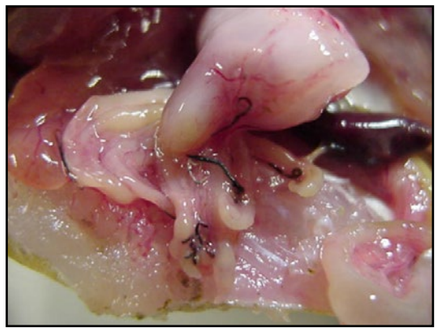


Figure 330. Nematode larvae in the peritoneum of yellow grouper (*Epinephelus awoara*).

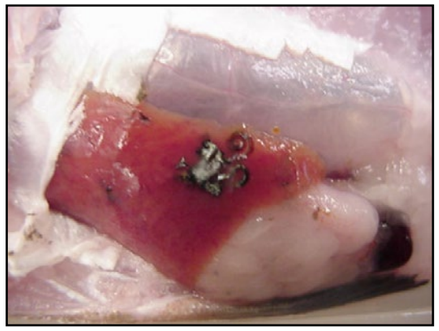


Figure 331. Nematode larval tracts in liver of brown spotted grouper (*Epinephelus areolatus*).

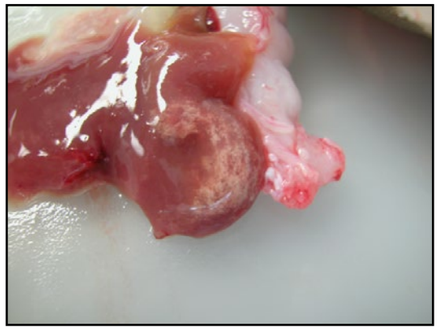


Figure 332. Scarring of liver from nematode larval migration in brown spotted grouper (*Epinephelus areolatus*).

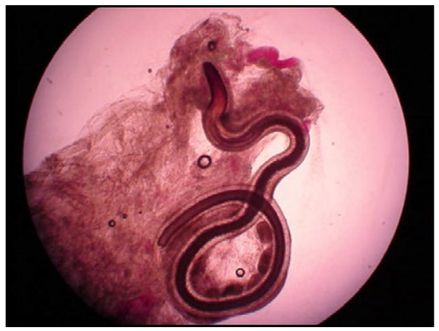


Figure 333. Wet mount of gut contents of coral grouper (*Plectropomus leopardus)* with a nematode adult.

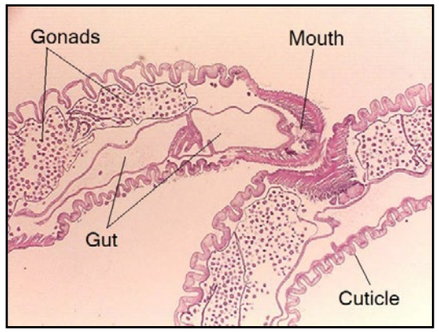


Figure 334. Distinguishing microanatomy of an adult nematode section in H&E.

Figure 335. Apparently healthy rabbit fish (*Siganidae* sp.) sampled from a retail market.

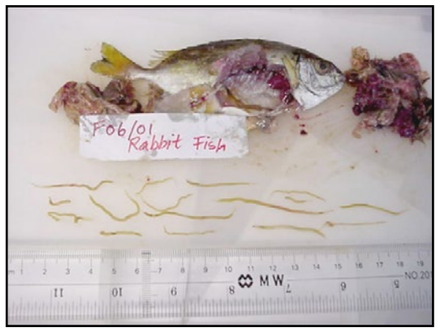


Figure 336. Up to 16 worms per fish indicating a heavy burden of nematodes in the intestinal tract of rabbit fish (*Siganidae* sp.).

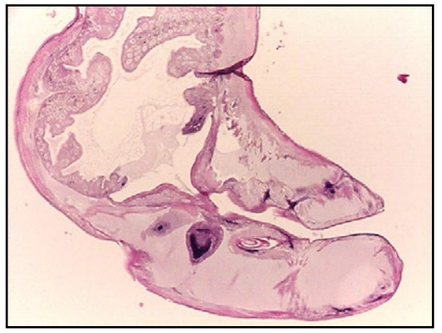


Figure 337. H&E section of the head end of a nematode found in rabbit fish (*Siganidae* sp.).

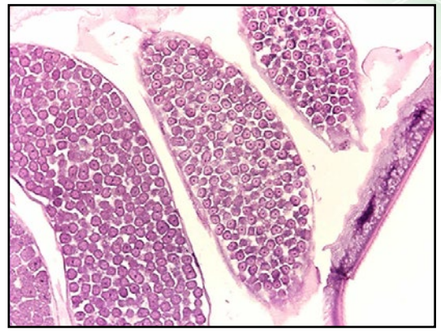


Figure 338. H&E cords of oocytes in the gonad of a nematode in rabbit fish (*Siganidae* sp.).

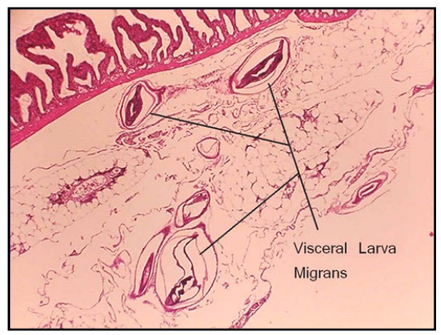


Figure 339. Sections of nematode larvae in the peritoneal fat of yellow grouper (*Epinephelus awoara*).

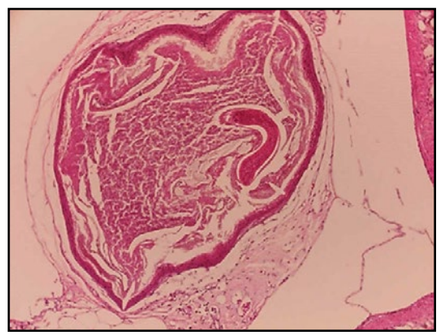


Figure 340. Nematode larval granuloma in yellow grouper (*Epinephelus awoara*).

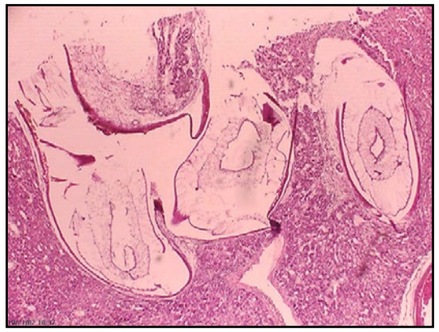


Figure 341. Nematode larval tracts in liver parenchyma of brown spotted grouper (*Epinephelus areolatus*).

Epidemiology

Nematoda have in-direct life cycles with intermediate hosts in which the larval stages of worms occur. These stages are usually situated outside the gut and may encyst until consumed by the definitive host. Definitive hosts contain the reproductive and adult stages usually in the lumen of the intestinal tract. Intermediate hosts can be a fish or invertebrate (crustacean) while definitive hosts can be a fish, bird or mammal including man. Eggs produced by adult worms are usually excreted via the faeces of the definitive host.

Pathophysiology

Nematodes in large numbers can result in negative energy balance of the host as the parasite out-competes the host for nutrients. Consumption of host blood leads to parasitic anaemia. This applies to adult stages. Larval stages can cause host tissue inflammation and damage through their migratory activities prior to encysting (visceral larva migrans).

Diagnosis

Identification of nematodes is achieved by the examination of the intestinal tract for adult stages and the rest of the internal organs for larval stages (Figs. 336-341). Grossly, tracts in the organs, e.g., the liver may indicate that visceral larva migrans is present. Definitive identification of the species of nematode requires proper collection and fixation of specimens and submission to a reference library, specialist parasitologist or PCR testing.

Control and Prevention

Control of nematodiasis is reliant on the application of anthelmintics, e.g., fenbendazole to infected batches of fish. It must be appreciated that this does not eradicate the helminthiasis but lowers the worm burden to levels that prevent negative health effects on the host. As far as zoonotic risks go for fish nematodes, *Anisakis simplex* has some public health risks from the consumption of infected, raw fish.

Cestodiasis

Farm History

Cestode helminths are occasionally detected in cultured marine fish and in wild caught fish. The level of infestation is generally low in cultured fish but can be significant in wild caught fish. Typically larval stages are observed rather than adult forms.

Clinical Signs

When a fillet of fish has cestode larvae in it, there may be associated some host reaction such as melanisation, which produces a blackened area adjacent to the normally flat and whitish cestodes (Figs. 342-343).

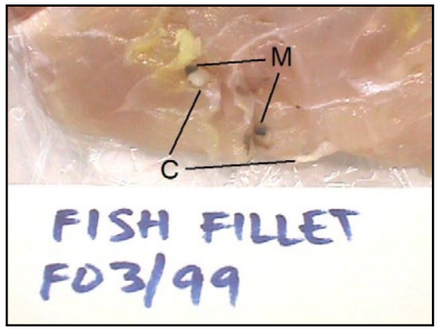


Figure 342. Grouper (*Epinephelus* sp.) fillet with cestode plerocercoid larvae (C) and associated melanisation (M).

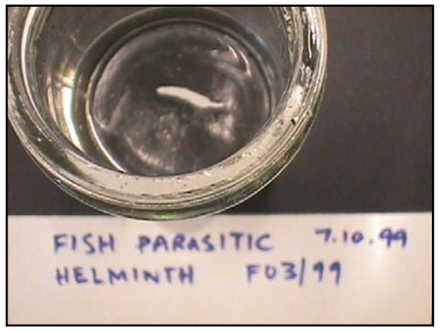


Figure 343. Dissected whitish cestode plerocercoid larva from the grouper (*Epinephelus* sp.) fillet.

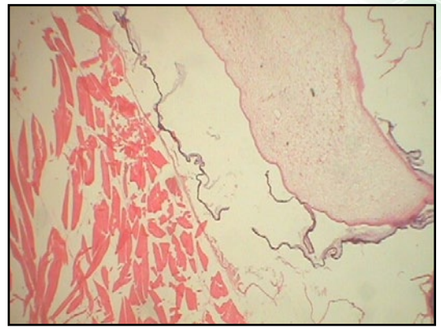


Figure 344. (H&E) Grouper (*Epinephelus* sp.) cestode plerocercoid larva from fillet; note the lack of internal gut structure as distinct from nematodes.

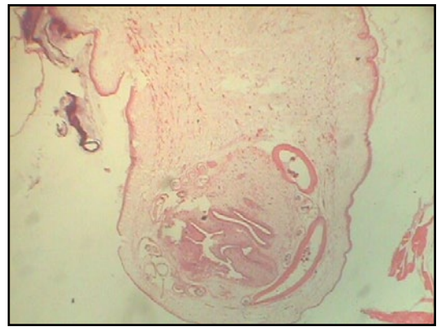


Figure 345. (H&E) Grouper (*Epinephelus* sp.) cestode plerocercoid larva showing the bothria of the anterior end.

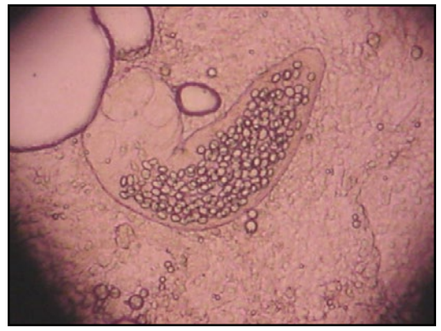


Figure 346. Cestode plerocercoid larva infective stage ingested into the intestine of snapper (*Lutjanus* sp.).

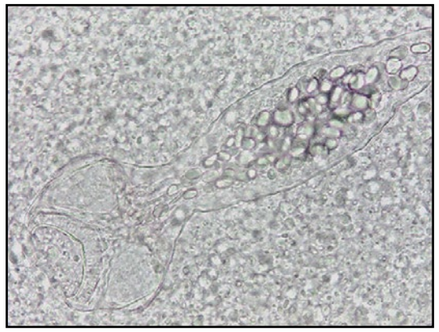


Figure 347. Cestode plerocercoid larva infective stage ingested into the intestine of grouper (*Epinephelus* sp.).

Epidemiology

The life cycle of cestodes involves the adult stage with a scolex and segmented proglottids producing eggs, which hatch into free-swimming coracidum. The coracidia develop into procercoids in an invertebrate, e.g., copepod intermediate host. Plerocercoid infective stages (Figs. 344-347) are released from the invertebrate to infect a second intermediate host, e.g., fish. These larval plerocercoid stages develop in the fish and when the fish is eaten by a fish, bird, mammal or shark, these become the definitive (adult reproducing) stages residing in the intestinal tract.

Pathophysiology

Cestodes in the adult stages compete with the definitive host for nutrient resources leading to host emaciation, poor growth and mortality. Larval cestodes migrate through the intermediate host tissues inducing localised pathology and inflammation. It is these plerocercoid stages, which cause the most damage in terms of carcass degradation in fish fillets.

Diagnosis

It is relatively straightforward to diagnose cestodiasis in fish by gross pathology and histopathology, recognising the flat morphology of the cestodes, which do not have an internal intestinal tract. However species identification requires fixation of the cestode and submission to a reference parasitology laboratory. Check with the parasitologist on the appropriate life-stage to send as adult cestodes are generally required and the larval forms in the fish samples may not allow full identification, apart from molecular (PCR) tests. Adult cestodes have detachable segments and a scolex attachment organ, are located in the intestinal lumen while larval plerocercoids have integumental folds and no scolex but are located in extra-intestinal tissues.

Control and Prevention

Cestodiasis is amenable to anthelmintic treatments using praziquantel active against the adult worm. Removal of infected intermediate hosts where possible can enhance control. Freshwater cestodes, e.g., *Diphyllobotrium latum* is a major zoonosis. The risk factors for diphyllobothriasis include the consumption of raw or marinated fish, particularly wild-caught fish prepared as sushi and sashimi, Scandinavian gravlax, strogonina in the Baltics and Eurasia, gefilte fish and ceviche in Latin America. Contamination of the local aquatic environment with faeces and fishermen who are in the habit of eating the roe and livers of their fresh catches also increase the risk of diphyllobothriasis.

Trypanosomiasis

Farm History

Trypanosomiasis caused by a marine *Trypanosoma* sp. was detected in marine brown spotted grouper (*Epinephelus areolatus*) with imported fish. Cumulative and chronic mortality of 25% was reported over 7 months.

Clinical Signs

Heavily infected fish may exhibit anaemia as indicated by a low haematocrit (15%) and low haemoglobin concentration (3.9 g/dL) and pale gills (Fig. 348). The gall bladder has been reported to be ruptured in some fish by farmers. The spleen is congested (Fig. 350) and enlarged while the liver is an off-brown colour (Fig. 349).

Figure 348. Brown spotted grouper (*Epinephelus areolatus*) with pale gills, indicating anaemia; trypanosomiasis.

Figure 349. Brown spotted grouper (*Epinephelus areolatus*) with off-brown liver; trypanosomiasis.

Epidemiology

Trypanosoma is transmitted by a biting marine leech. A single leech species can often transmit more than one trypanosome species. They develop in the gut of the leech, producing large numbers of the fish infective stage (trypomastigotes), which are then transferred to a fish host when the leech bites and feeds on the fish's blood. Trypomastigotes in fish blood may be small (acute infection) or large (chronic infection) since they increase in size the longer they remain in the blood. There may be a mixture of forms in the blood due to repetitive infections by leeches. When ingested by a leech, the trypomastigotes form amastigotes, which have no flagellum. The stage then goes through several developmental stages before becoming a trypomastigote, which is infective for other fish.

Figure 350. *Epinephelus areolatus* with congested spleen; trypanosomiasis.

Pathophysiology

Trypanosomes infiltrate all organs and their large numbers, feeding and activity can damage erythrocytes. The primary disease is a type of haemolytic anaemia (destruction of red blood cells) due to heavy infection. Anaemic fish are more sensitive to the stress of handling and any low oxygen levels in the water.

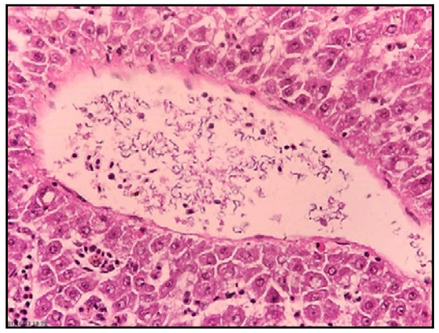


Figure 351. *Trypanosoma* sp. trypomastigotes in liver vein of brown spotted grouper (*Epinephelus areolatus*).

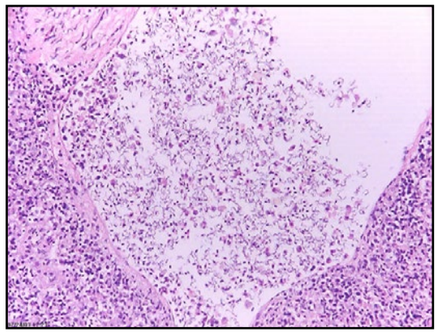


Figure 352. *Trypanosoma* sp. trypomastigotes in spleen of brown spotted grouper (*Epinephelus areolatus*).

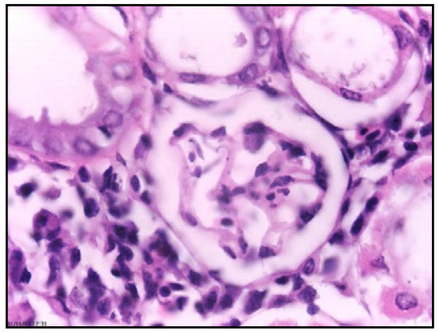


Figure 353. *Trypanosoma* sp. trypomastigotes causing glomerular dilatation in kidney of brown spotted grouper (*Epinephelus areolatus*).

Diagnosis

Wet mounts of gills, peritoneal and kidney smears allow identification of the haemoflagellates by observing their flagellum and wriggling motion (Figs. 354-356). Giemsa stained blood smears enable morphological examination. Each trypomastigote possesses a single flagellum, a well-developed undulating membrane, a kinetoplast and a nucleus (Fig. 355), which are consistent with *Trypanosoma* sp. (Stoskopf, 1993). The trypomastigotes measure approximately 30 μm (including the flagellum) in length. The trypomastigotes appear monomorphic and are of a size suggestive of recent infection (Chong, 2005). Histologically there are numerous trypomastigotes in the major vessels of the liver, spleen and also in smaller vascular spaces, e.g. renal corpuscle (Figs. 351-353).

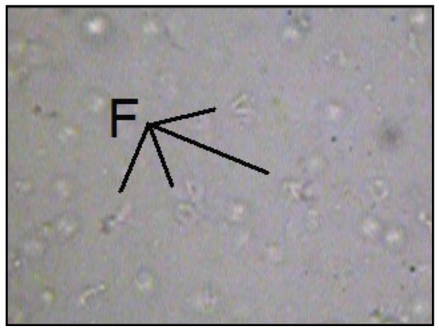


Figure 354. Wet mount showing haemoflagellates (F).

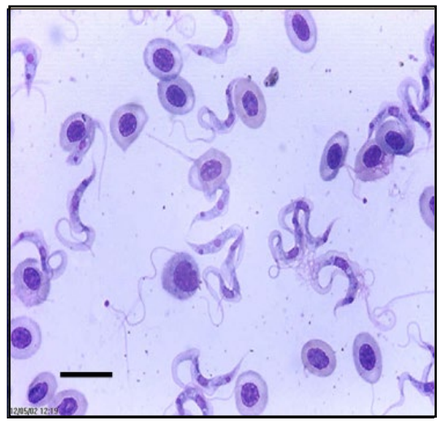


Figure 355. *Trypanosoma* sp. trypomastigotes in blood smear of brown spotted grouper (*Epinephelus areolatus*); scale bar = ~10 μm; Giemsa stained peripheral blood smear x 1000.

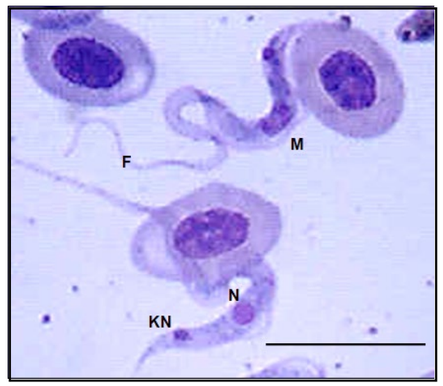


Figure 356. *Trypanosoma* sp. trypomastigotes of brown spotted grouper (*Epinephelus areolatus*); morphological features: a single flagellum (F), well-developed undulating membrane (M), a kinetoplast (KN) and a nucleus (N); scale bar = ~ 10 μm; Giemsa stained peripheral blood smear x 3500.

Control and Prevention

Treatment recommended to farmers is the control of leeches with 2 ppm trichlorphon bath for 60 minutes in seawater. Isometamidium chloride (a trypanocidal drug) used at 1.0 mg/kg intramuscularly is reportedly effective against a related haemoflagellate *Cryptobia salmositica* in Chinook salmon (Woo, 2001). An antimalarial drug - pyrimethamine is reportedly effective against *Cryptobia* spp. (Herwig, 1979).

There is currently no known public health risk with piscine trypanosomiasis (Stoskopf, 1993). Due to the small size of the trypomastigotes and the delayed onset of mortalities some months after importation of the fish, it is likely that the source of the parasite is local. However, survey studies of imported and indigenous marine fish in Hong Kong are needed to provide necessary information on the type and distribution of tropical and sub-tropical piscine trypanosome species. This will enable a risk assessment of the health impact of these haemoparasites on cultured marine fish.

Trichodiniasis

Farm History

Trichodinids are commonly observed in marine and freshwater fish. They are generally a reflection of the level of hygiene and effective husbandry of a farm. Large numbers of trichodinids generally indicate poor hygiene and husbandry. This usually leads to other more serious disease conditions such as additional parasites and bacterial disease.

Clinical Signs

While *Trichodina* spp. inhabit the skin and gills, it is their density on the gills, which causes problems for fish. The excessive mucus production may impair gill function. Clinical signs of other diseases usually dominate the clinical presentation rather than that caused by the trichodinids themselves.

Epidemiology

Trichodinids reproduce by binary fission; hence numbers can escalate quickly on fish that are stressed. In organically polluted conditions and with high stocking density, trichodinids can proliferate. Low temperatures and depressed fish immunity may favour the establishment of trichodiniasis.

Pathophysiology

While trichodinids tend not to be firmly attached to host epithelia, the feeding activity can irritate gills and skin leading to hyperplasia and mucus production. These ciliates are mobile and are observed to 'graze' on the gills and skin.

Diagnosis

A wet mount of the gills and skin will readily detect the presence of circular, saucer-shaped ciliates that rotate and glide. The denticular morphology of the sucking or adhesive disc is used to differentiate to the species level. Laterally they have a semi-ovoid shape (Figs. 357-360). Diagnosis to the species level requires staining with silver nitrate impregnation technique. Measurements of the morphometrics of the adhesive disc, the denticulated ring, number of denticles, span and length of denticles are compared to a library of some 300 *Trichodina* species. Recently described *Trichodina* species in common carp, *Cyprinus carpio,* is *Trichodina cyprinocola* and from goldfish, *Carassius auratus,* is *T. borokensis* (Zhao and Tang, 2011).

Figure 357. Trichodinid feeding on gill surface of brown spotted grouper (*Epinephelus areolatus*); lateral view of ciliate.

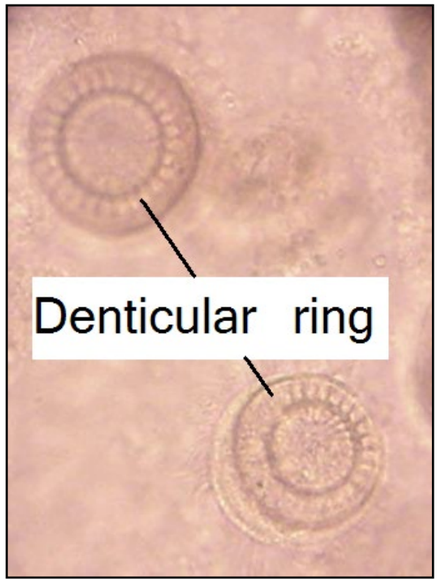


Figure 358. Goldfish (*Carassius auratus*) trichodinids, ventral view of adhesive disc.

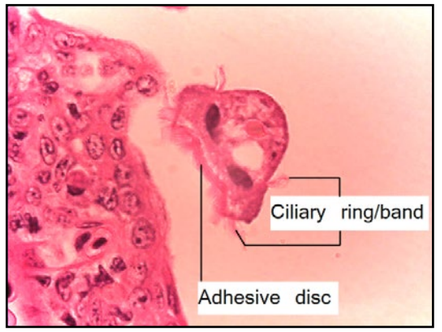


Figure 359. H&E section of trichodinid associated with hyperplastic gill lamellae.

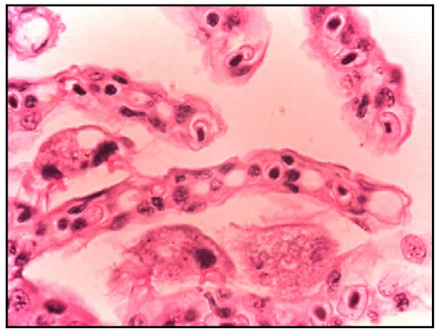


Figure 360. H&E section of trichodinids showing feeding activity on the gill lamellae.

Control and Prevention

Marine trichodinids require freshwater bathing and freshwater species are controlled with salt baths. Husbandry improvements include:

- Reduction of stocking density
- Improving water exchange
- Supplementary aeration

Concomitant parasites such as gill flukes, *Benedinia*, *Brooklynella* and bacterial infections must be treated at the same time.

Sanguinicoliasis

Farm History

Sanguinicola sp. is a digenean trematode (fluke) occasionally seen in marine brown spotted grouper (*Epinephelus areolatus*). Disease and mortalities are usually associated with other parasites and bacterial infection.

Clinical Signs

Sanguinicola sp. eggs localise in the gill filaments and may block blood flow. Infected fish may show pale gills and areas of haemorrhage, melanisation and necrosis. Concurrent parasites such as *Dactylogyrus*, *Trichodina* or *Benedinia* may produce similarly pale gill lesions (Fig. 361).

Figure 361. Brown spotted grouper (*Epinephelus areolatus*) with pale gills, ecchymotic haemorrhages and necrosis across the same portions of the gill arches suggestive of ischaemic effects due to *Sanguinicola* eggs.

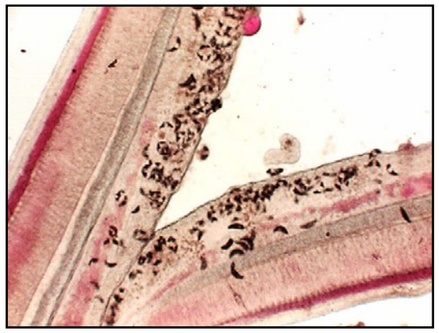


Figure 362. *Sanguinicola* fluke eggs in the gill filament of brown spotted grouper (*Epinephelus areolatus*).

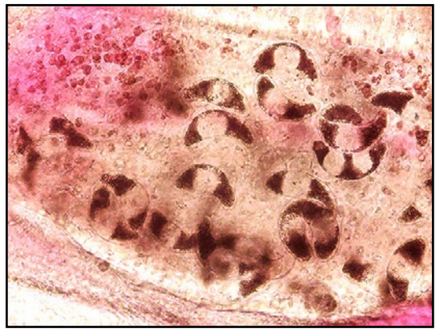


Figure 363. *Sanguinicola* fluke eggs with a 'cashew nut' shape lodged in the gill filament.

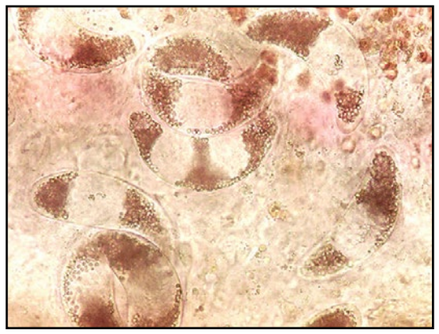


Figure 364. *Sanguinicola* fluke eggs showing the opaque ends and the clear central portion.

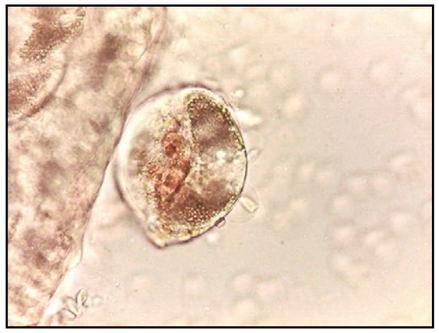


Figure 365. *Sanguinicola* fluke egg undergoing embryonation.

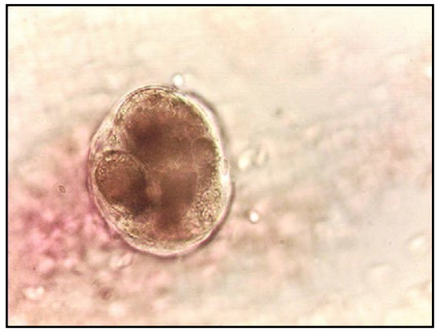


Figure 366. Embryonated *Sanguinicola* fluke egg, with miracidium form, prior to release from the egg capsule.

Epidemiology

The life cycle of marine *Sanguinicola* sp. involves a polychaete rather than a molluscan intermediate host. Cercariae infect the definitive fish host, and develop into adults within the blood vessels. Eggs are released in the vascular system and these usually get lodged in the capillary system of the gills. The eggs may develop into miracidia in the gills with subsequent release into the water upon gill damage (Figs. 362-366). Only three sanguinicolid have been described from grouper species – *Adelomyllos teenae* in *E. coioides, Pearsonellum pygmaeus* in *Cromileptes altivelis* and *P. corventum* in *Plectropomus leopardus* (Nolan and Cribb, 2004).

Pathophysiology

Ischaemic thrombosis, haemorrhagic anaemia with gill hyperplasia, fusion and/or necrosis are contributive to fish mortalities (Fig. 369). Local cases have had gill egg count estimates of 60, 971 and 35,000 per fish. Gill necrosis and haemorrhage have been noted in fish gills with an estimated count of 60 eggs but not in fish with 971 or 35,000 eggs. Nevertheless, it is probably the location of the eggs within the gill vascular tree, as well as the numbers of eggs, which determines whether lamellar ischaemic thrombosis and necrosis occur.

Diagnosis

Wet mount examination of the gills readily identifies cashew nut-shaped eggs showing tapered ends. The ends are opaque and the centre is more translucent. Adult flukes may be detected by histopathology (Figs. 367-368).

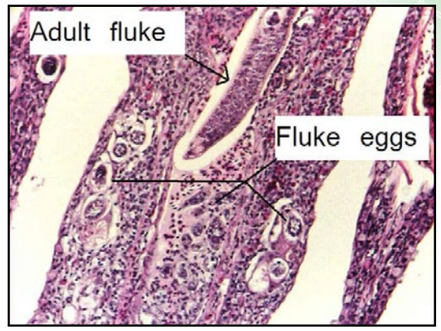


Figure 367. Severe gill hyperplasia and fusion; *Sanguinicola* adult with eggs of brown spotted grouper (*Epinephelus areolatus*).

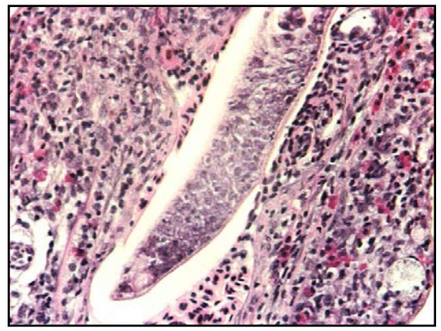


Figure 368. Adult *Sanguinicola* fluke occluding the lamellar capillary of brown spotted grouper (*Epinephelus areolatus*).

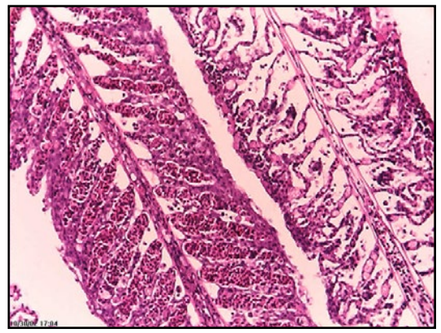


Figure 369. Lamellar vascular stasis, haemorrhage and necrosis; sanguinicoliasis in brown spotted grouper (*Epinephelus areolatus*).

Control and Prevention

Praziquantel at 150 mg/kg fish once a day for three treatments is prescribed along with control of associated parasites and bacterial infection where these occur. The species of *Sanguinicola* in *E. areolatus* appears to be host specific as cross-infestation of other grouper or marine species has not been observed.

Myxosporidiosis

Farm History

Myxosporeans commonly occur in goldfish and occasionally in marine fish. *Myxobolus* sp. and *Thellohanellus* sp. occur in the gills and muscle while *Sphaerospora* sp. targets the kidney of goldfish. Actinosporean infective stages occur on the gills of black seabream (Figs. 371, 386), and *Sphaerospora* sp. occurs in the kidney tubules of *E. areolatus*. Levels of infestation range from low to moderate and are seldom associated with fish mortalities.

Clinical Signs

For goldfish, white nodules or cysts are visible on the gills (Fig. 370). These are much larger than white spot trophonts. Some thickening and excess mucus may be noted in moderately infected fish. For marine fish, gross signs of infection are not evident as infections are usually of a low level.

Figure 370. Goldfish (*Carassius auratus*) with myxosporean plasmodia on the gills.

Epidemiology

Myxosporeans have indirect life cycles, involving the release of spores from the fish definitive host, which are then taken up by an annelid (worm) intermediate host. Actinosporean stages emerge from the annelid host to infect fish. At the target tissue, plasmodia develop and enlarge, becoming spore forming structures in the fish. Recently the myxosporeans have been considered metazoans because some species are multicellular.

Pathophysiology

Infected fish often contain variable numbers of active plasmodia. In the gills, the plasmodia displace gill lamellae and can incite hyperplastic responses (Figs. 375, 378). Lamellar fusion may occur. Severe infections lead to gill distortion (Figs. 376, 383 and 384). In the kidney tubules, spores and pseudoplasmodia can occlude the normal flow of glomerular filtrate leading to dilatation and degeneration of tubules with glomerular necrosis (Figs. 379-380, 385). Inflammatory responses are generally mild around the intact plasmodia. Rupture of gill plasmodia leads to the release of spores into the external environment (Fig. 377). *Sphaerospora* sp. spores may be released from the urinary tract. Muscle located myxosporeans (Fig. 381) become a problem after the fish dies due to the release of enzymes that are potentially liquefactive in muscle, e.g., *Kudoa* sp.; however this is not observed for the species of myxosporeans observed locally.

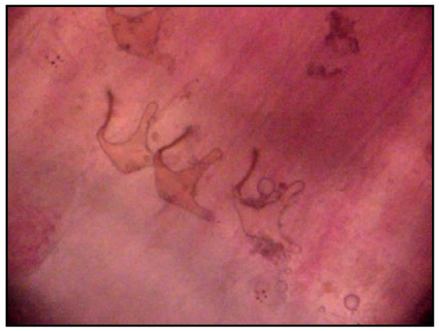


Figure 371. Actinosporean infective stages of myxosporean in black seabream; note the three-pronged structure, enabling attachment to the gills.

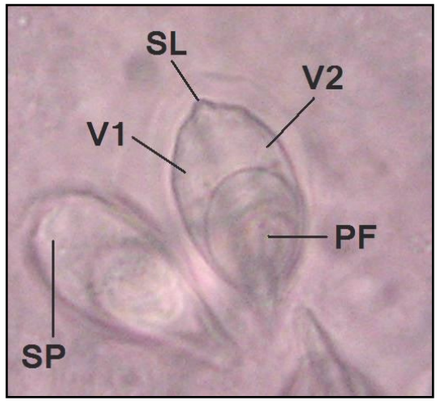


Figure 372. *Thellohanellus* sp. spores: suture line (SL), valve 1 (V1), valve 2 (V2), polar filament (PF) and sporoplasm (SP). Wet mount from gills of goldfish (*Carassius auratus*).

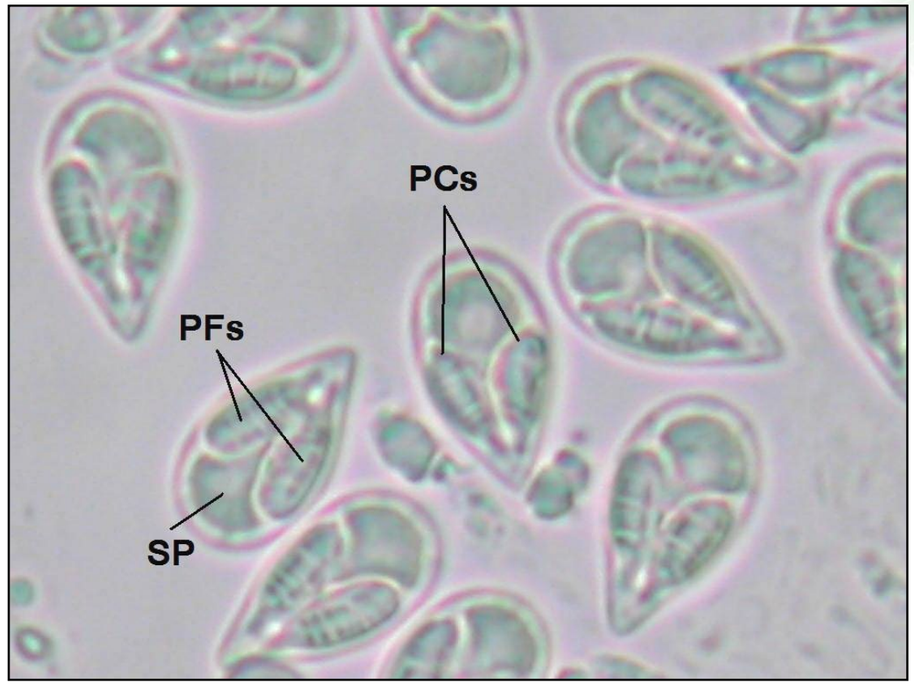


Figure 373. *Myxobolus* sp. spores; note the two polar filaments (PFs) inside two polar capsules (PCs) and sporoplasm (SP). Wet mount from gills of goldfish (*Carassius auratus*).

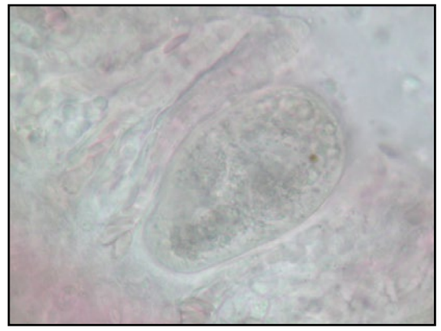


Figure 374. Wet mount of plasmodium from gills of goldfish (*Carassius auratus*); note the displacement effect on lamella.

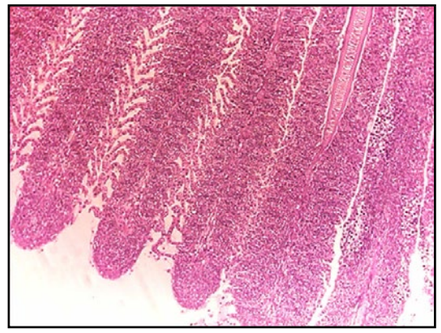


Figure 375. Severe gill hyperplasia and fusion due to *Myxobolus* sp. infection; goldfish (*Carassius auratus*).

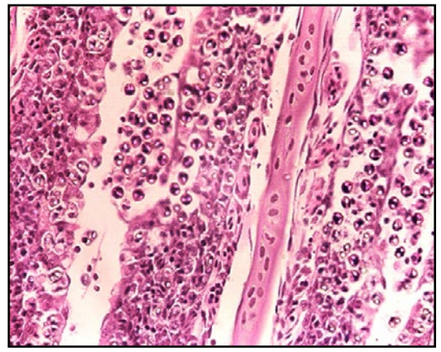


Figure 376. *Myxobolus* sp. spores infiltrating and replacing normal gill lamellar structure; note the refractile polar capsules; goldfish (*Carassius auratus*).

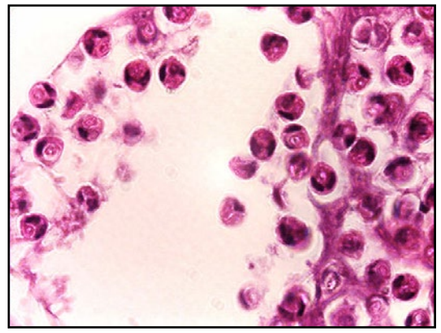


Figure 377. Release of *Myxobolus* sp. spores from the damaged gill surface; goldfish (*Carassius auratus*).

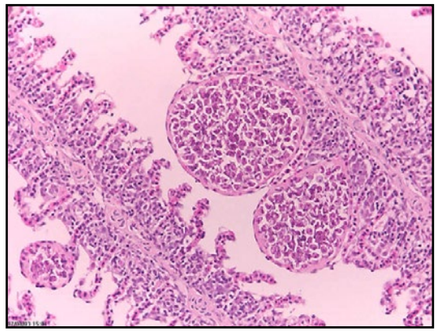


Figure 378. Myxosporean plasmodia and generalised lamellar hyperplasia; note the integration of the parasite into host tissue.

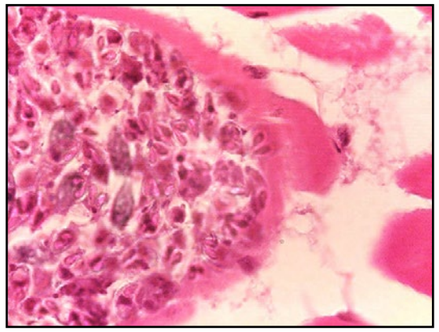


Figure 381. *Myxobolus* sp. spores in muscle of goldfish (*Carassius auratus*).

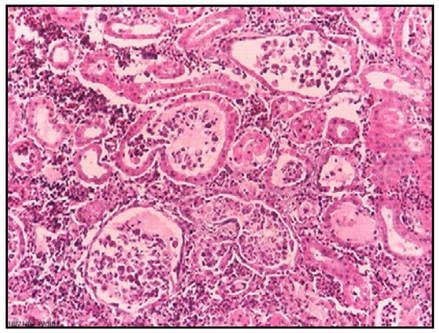


Figure 379. Renal tubular dilation and degeneration due to sporocysts and spores of *Sphaerospora* sp. in brown spotted grouper (*Epinephelus areolatus*).

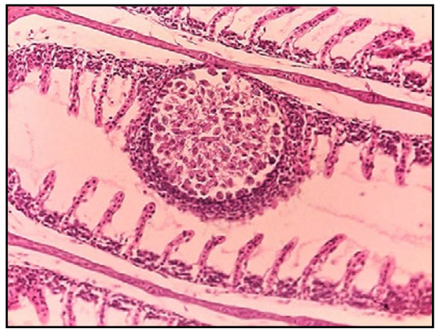


Figure 382. *Myxobolus* sp. plasmodium in goldfish (*Carassius auratus*) gills; note the hyperplasia localised to the plasmodium. Contrast this with Fig. 383.

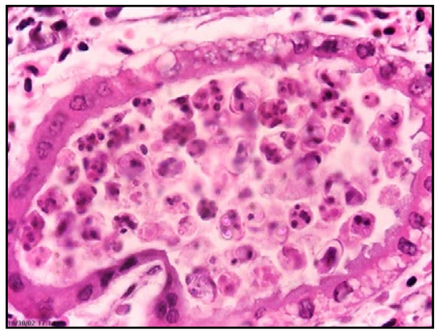


Figure 380. Spores of *Sphaerospora* sp. as a pseudoplasmodium occluding the renal tubule in brown spotted grouper (*Epinephelus areolatus*).

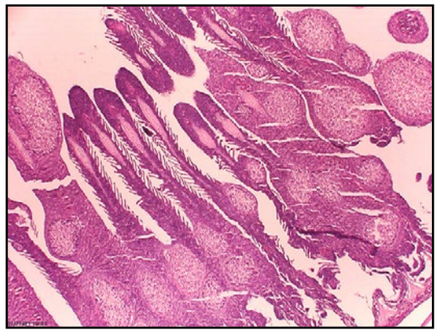


Figure 383. *Myxobolus* sp. plasmodia causing lamellar hyperplasia and distortion in goldfish (*Carassius auratus*).

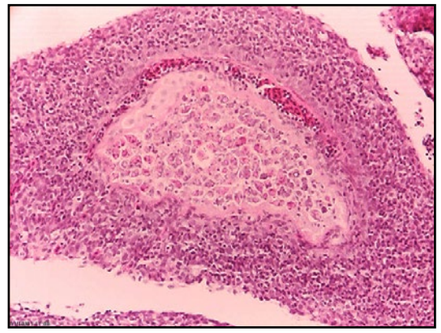


Figure 384. Plasmodium of *Thellohanellus* sp. in gills of goldfish (*Carassius auratus*).

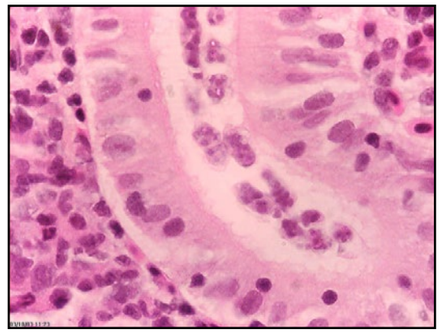


Figure 385. *Sphaerospora* sp. spores in the renal tubule of goldfish (*Carassius auratus).*

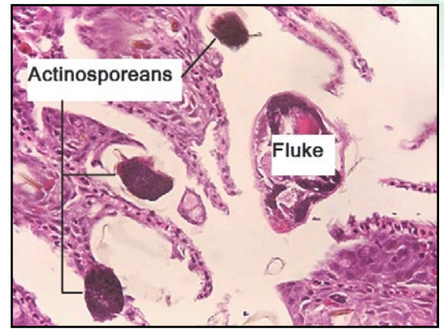


Figure 386. Actinosporean stages of a marine myxosporean infecting the gills of black seabream (*Spondyliosoma cantharus*).

Control and Prevention

There is no effective medication for infected fish although the literature reports that fumagillin has been tried with variable effect in some myxosporeans but none in others (Woo, 1995). Means of control would therefore need to focus on:

- Elimination of infected fish
- Exclusion of potential contact with annelid intermediate hosts
- Disinfection of premises

Diagnosis

Wet mount examination of squashed white gill nodules readily reveals the presence of spores. Myxosporean spores have varied morphology but typically consist of one or more refractile polar capsules, one or more valves, a suture line and a posterior compartment containing the sporoplasm. Myxosporeans are multicellular and likely to be metazoa rather than protozoa. Table 37 shows the distinguishing features of local myxosporeans. Giemsa or Ziehl-Neelsen staining will highlight the polar capsules in histological section.

Table 37. Features of Myxosporean spores

Myxobolus sp.	Ovoid egg-shaped, 2 polar capsules, suture line, 2 valves
Thellohanellus sp.	Pyriform, single polar capsule, suture line, 2 valves
Sphaerospora sp.	Spherical, 2 polar capsules, suture line, 2 valves

Infection with *Argulus*

Farm History

The crustacean *Argulus* (or fish louse) is a freshwater parasite observed occasionally on the skin of goldfish and koi carp. Its importance in these fish species is the potential ability to act as vectors for Spring Viremia of Carp Virus (SVCV). While SVCV has not been detected in Hong Kong (1999 – 2003), control of *Argulus* infestation on goldfish and koi is an important aspect of risk management should SVCV occur.

Clinical Signs

Infected fish where many fish lice are present may cause skin ulcerations due to the piercing activity by the parasite and secondary self-trauma of the fish. Skin areas may be haemorrhagic and lead to secondary infections. Fish may exhibit flashing, or rubbing. As fish lice suck blood from the host, anaemia may develop from high and chronic levels of lice (Figs. 387 -388).

Figure 387. Koi carp (*Cyprinus carpio*) with skin haemorrhages.

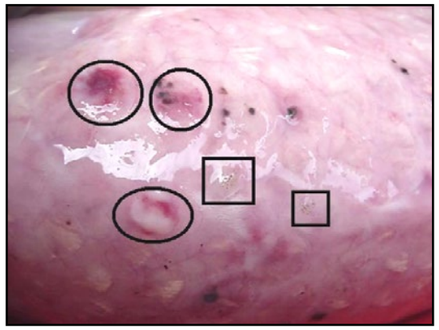


Figure 388. *Argulus* (rectangles) and probable bite marks (circular) from *Argulus*; koi carp (*Cyprinus carpio*).

Epidemiology

Argulus reproduces in a direct life cycle where eggs develop into sexually mature lice. The optimum temperature for *Argulus* is 10ºC – 28 ºC (Brown, 1993). Under conditions of crowding and sluggish water exchange in a fish pond, populations of *Argulus* can rapidly increase. New lice can begin parasitising fish in 4 weeks post-hatching and be reproducing in 5-6 weeks after feeding on fish (Stoskopf, 1993).

Pathophysiology

Argulus has a piercing mouthpart used to puncture the skin of fish in order to extract a blood meal. The production of toxic enzymes (anticoagulant) can cause localised inflammation, haemorrhage and necrosis. It is this stylet mouthpart, which can be contaminated with virus, bacteria or even haemoflagellates. Thus *Argulus* can be a vector for disease. *Argulus* attaches onto the host by its sucking discs, and it is freely mobile on the fish, changing feeding site as required.

Diagnosis

Argulus lice are about 5 mm in length. They are flat and disc-shaped, with four pairs of legs, and have two eyes, two sucking discs on the ventral surface and a short tail of two flaps. They, being almost transparent, may camouflage themselves on the skin of fish (Fig. 390).

Control and Prevention

Control methods include one of the following chemical baths:

- Potassium permanganate
- Organophosphate
- Formalin

In addition, heavily infested ponds may need to be drained and limed, in order to remove laid eggs and recent hatchlings of lice (Fig. 389).

Figure 389. An urban ornamental fish pond being drained and cleaned; note the high algal and organic content, which is conducive to *Argulus* proliferation.

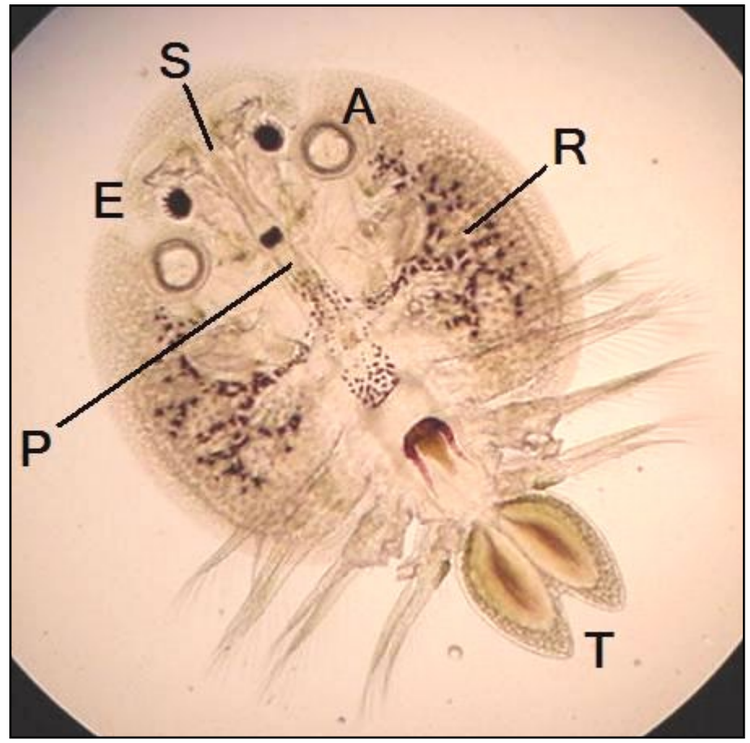


Figure 390. *Argulus* wet mount: stylet (S), proboscis (P), eye (E), attachment sucker (A), respiratory organ (R) and tail (T).

Coccidiosis

Farm History

Coccidiosis is fairly common in goldfish, and is occasionally observed in marine grouper and koi carp. Coccidial infections are generally subclinical in goldfish but can cause mortalities in grouper.

Clinical Signs

Heavy infections and the release of sporozoites within oocysts result in intestinal inflammation, necrosis with mucoid faeces and fish mortalities. Chronic infections may produce emaciation, lethargy and inappetence (Brown, 1993). Only intestinal coccidiosis has been detected in local fish but the literature reports extra intestinal types of coccidiosis (Noga, 1996). The clinical signs for systemic coccidiosis are dependent on the organ(s) affected (Figs. 391-392).

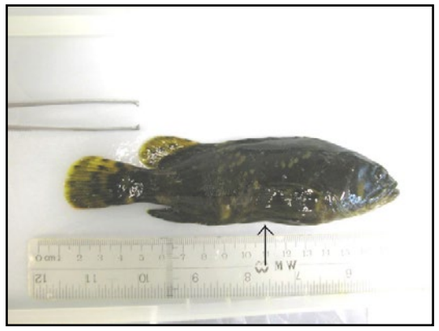


Figure 391. Giant grouper (*Epinephelus lanceolatus*) with coccidiosis showing poor body condition; note the shrunken abdominal profile (arrow).

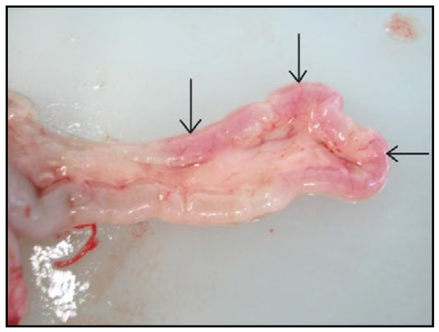


Figure 392. Congested and inflamed intestine; giant grouper (*Epinephelus lanceolatus*) with coccidiosis.

Epidemiology

Fish coccidia primarily belong to the Eimeriidae family. The life cycle is complex. *Eimeria* oocysts contain four sporocysts within which are two sporozoites. Upon release into the intestinal lumen, the oocyts excyst to release the sporocysts. Sporocysts release sporozoites, which become infective for other fish. For intestinal coccidia, the sporozoites enter intestinal epithelial cells to begin the direct life cycle. The sporozoite undergoes merogony to form a multinucleate meront stage containing merozoites (asexual reproduction). The merozoites then infect another host cell to undergo either another merogony cycle or sexual reproduction called gametogony. Gametogony can produce either macrogametes or microgametes. These gametes produce zygotes. Under sporogony, zygotes then produce oocysts, which have developing sporocysts that are then passed into the faeces.

Pathophysiology

In intestinal coccidiosis, the host impact is based on:

- A reduction in digestive and absorptive functions of the gut as a result of the reproductive activities of the coccidia causing gradual loss of functional intestinal epithelial cells. The initial host response is simple replacement of intestinal cells (Woo, 1995).
- Intestinal inflammation and epithelial sloughing with mass release of oocyst stages (Woo, 1995).

Diagnosis

Faecal wet mount is used for the detection of oocyst stages and histology for the detection of various stages of coccidia in the intestine. Note any associated intestinal inflammation or sloughing. Typically the merogony, gametogony and sporogony stages are mainly located in the lamina propria underneath the epithelial lining prior to rupture and oocyst release. A few oocysts may work their way into the epithelium. Usually up to three sporocysts are visible in H&E sections (Figs. 393-402).

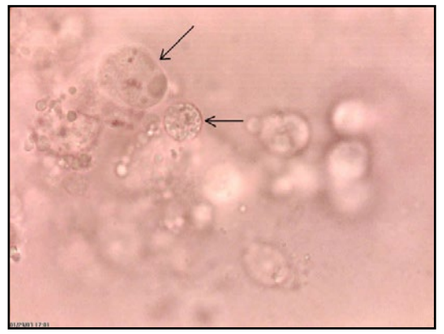


Figure 393. Unsporulated oocysts of coccidia from goldfish (*Carassius auratus*) faecal smear.

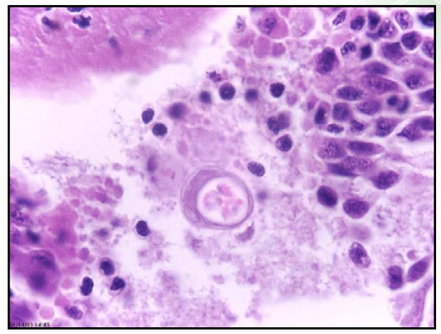


Figure 396. Coccidiosis in goldfish; released oocyst with an amount of host tissue (yellow body) surrounding the oocyst, which contains sporocysts.

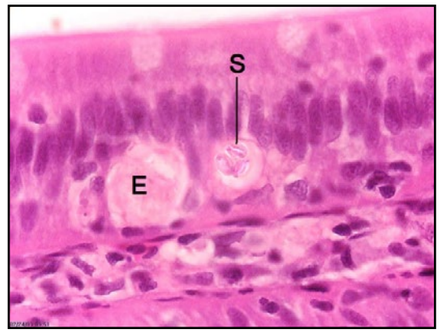


Figure 394. Coccidiosis in goldfish (*Carassius auratus*) intestinal epithelium; oocyst with three visible sporocysts (S), each with two sporozoites; empty gap (E) in epithelium due to released oocyst.

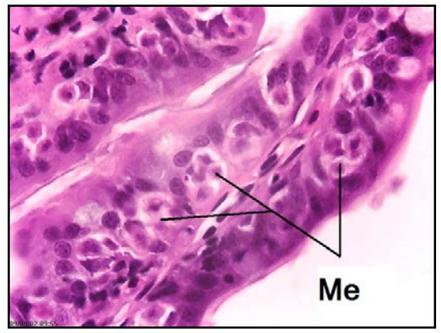


Figure 397. Coccidiosis in goldfish (*Carassius auratus*); meront stages (Me).

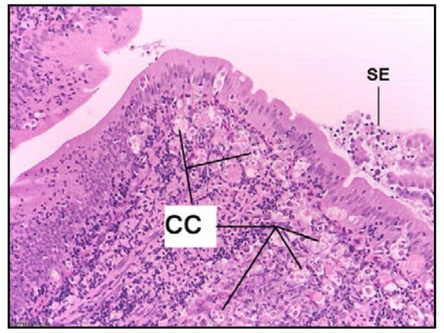


Figure 395. Coccidiosis in goldfish (*Carassius auratus*) intestine; note the sloughed epithelial cells (SE) and numerous subepithelial oocysts (CC).

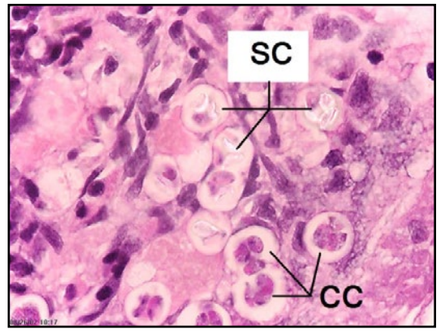


Figure 398. Intestinal coccidiosis in goldfish (*Carassius auratus*); sporulated oocysts (SC), developing and unsporulated oocysts (CC).

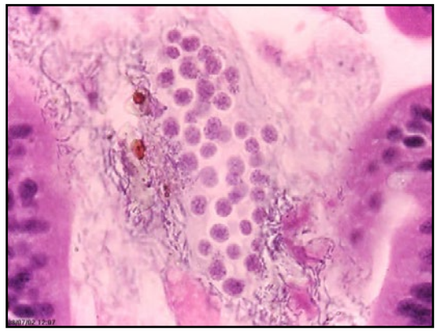


Figure 399. Coccidial oocysts in the lumen of goldfish (*Carassius auratus*) intestine; non-sporulated.

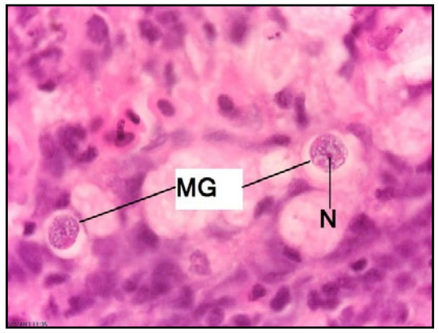


Figure 400. Coccidiosis in goldfish (*Carassius auratus*) intestine; macrogamete stages (MG) – note the central nucleus (N).

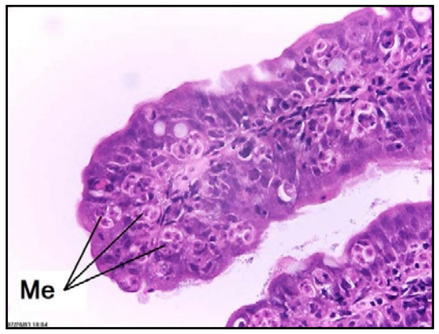


Figure 401. Giant grouper (*Epinephelus lanceolatus*) with intestinal coccidiosis; meronts (Me).

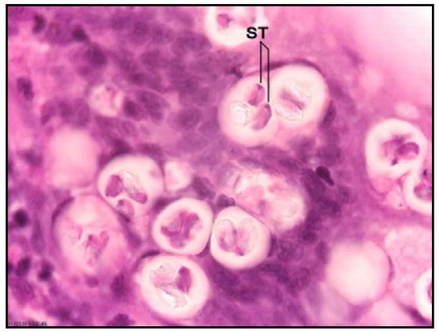


Figure 402. *Epieimeria* sp. (Family Eimeriidae) in goldfish (*Carassius auratus*). Oocysts with sporocysts; note steida body (ST) of the sporocysts. Usually up to three sporocysts per oocyst are visible in H&E sections.

Control and Prevention

Coccidial treatment recommended is a toltrazuril bath at 20 mg/L for 2 hours per day for 3 days. Experimental treatment of *Goussia carpelli* in common carp fingerlings suggest that diclazuril, lasalocid, robenidine HCl or maduramicin were free from infection by the coccidium 14 days after exposure but fish treated with amprolium, toltrazuril, monensin Na, narasin or salinomycin Na still had oocysts in the mucus and epithelium of the gut (Molnár and Ostoros, 2007). It should be noted that none of these drugs are currently registered for use in food species and regulatory permission should be sought prior to use as fish treatment, keeping in mind fish residue and environmental constraints. For pond culture, pond hygiene, drying and liming is necessary for sustainable control.

Oodiniasis

Farm History

Oodinium are dinoflagellates that parasitise freshwater fish such as goldfish, neon tetra, flame tetra, albino cory and black skirt. In heavy infestations, up to 80% morbidity and 50% mortality has been reported.

Clinical Signs

Typically called 'velvet or gold-dust disease', oodiniasis presents as a skin and gill condition. Infected fish display a dusty or velvety look on the skin, excessive mucus, laboured breathing, skin erosions and inappetence. The colour of the 'dust' varies from yellow to green (Figs. 403-404).

Figure 403. Black moor goldfish (*Carassius auratus*) with oodiniasis; note the greenish specks on the head and gill covers.

Figure 404. Black moor goldfish (*Carassius auratus*) with oodiniasis; note the greenish specks on the body, fins and tail.

Epidemiology

Oodinium infests fish as free-swimming dinospores. Once on the fish, trophonts develop. Trophonts leave the fish and become tomonts. Tomonts produce dinospores. The life cycle is completed in 10 – 14 days at 25ºC but is extended at lower temperatures. Dinospores infect fish for up to 48 hours (Noga, 1996).

Pathophysiology

Host damage is done by the feeding and lysis of epithelial cells in the gills and skin of heavily infested fish. The trophonts develop rhizoids (roots) for attachment to host tissues. Hydrodynamic forces of the 'hanging' trophonts enable the fragmentation of host cells as the fish swim (Woo, 1995). The irritation and cell damage incites hyperplasia and fusion (clubbing) of the gill lamellae (Figs. 405-406) or necrosis in severe cases.

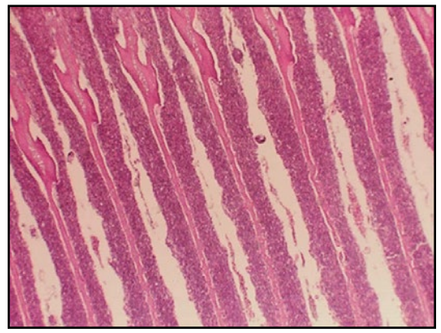


Figure 405. Gill lamellar fusion (clubbing) due to chronic hyperplasia from oodiniasis; goldfish (*Carassius auratus*).

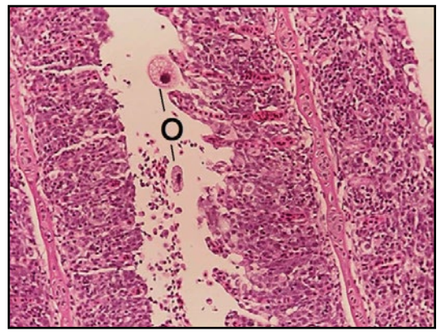


Figure 406. Goldfish (*Carassius auratus*) gills with oodiniasis, severe hyperplasia and fusion.

Diagnosis

Wet mount preparations of gill and skin are used to identify the trophont stages which are typically pyriform to oval in shape and contain chloroplasts with chlorophyll. The greenish colour depends on the amount of chlorophyll present in the trophont. There are also rounded vacuoles inside the cell wall of the trophont. The rhizoids or roots may be visible attaching to the host tissue (Figs. 407-411).

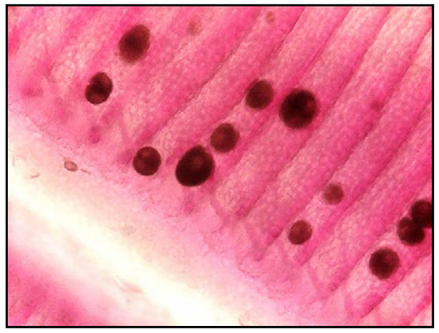


Figure 407. Trophonts of *Oodinium*; goldfish (*Carassius auratus*) gills.

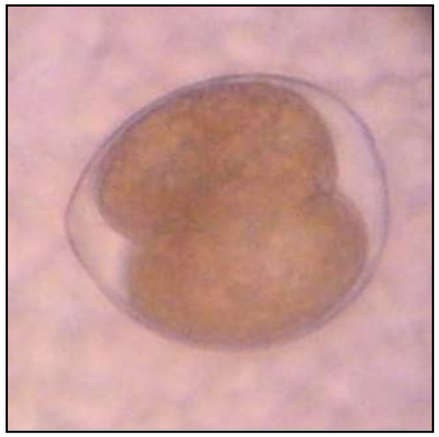


Figure 408. Dividing tomont in the process of dinospore production once released from the fish.

Control and Prevention

For heavy infestations, a salt dip at 35 ppt is needed to quickly dislodge trophonts, followed by a 5 ppt prolonged salt immersion. Pond hygiene to remove accumulated organic wastes and drying are necessary for the prevention of reinfestations.

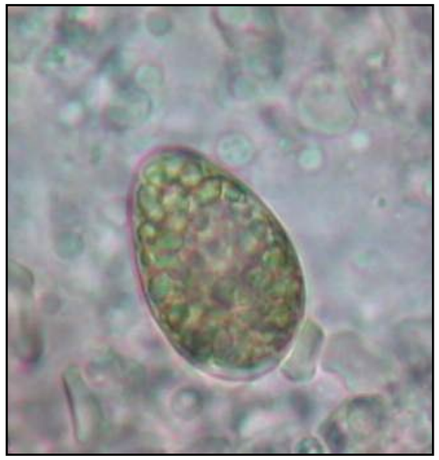


Figure 409. Immature pyriform-shaped trophont with green chloroplasts and rounded vacuoles; note the prominent cell wall.

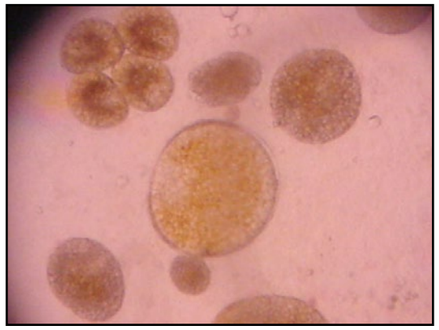


Figure 410. More yellow pigmented trophonts of *Oodinium* in goldfish (*Carassius auratus*)

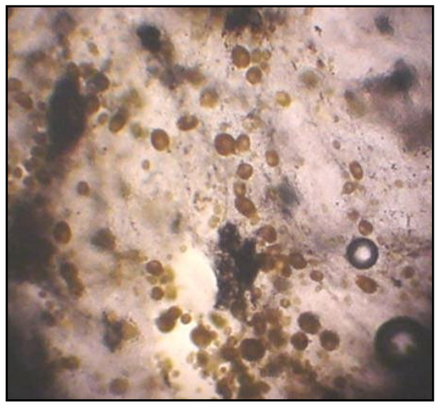


Figure 411. Skin scraping revealing greenish *Oodinium* trophonts.

Apiosomiasis

Farm History

Ectocommensal protozoa can be associated with pond culture of fish. They are in general an indicator of the level of organic loading in the pond environment. Heavy infestation on fish can result in mortalities.

Clinical Signs

Milk fish *(Chanos chanos)* fry infected (Fig. 412) by apiosomiasis displayed a cumulative mortality rate of 26% in 5 days. Mortalities began at 50-100 tails daily for 3 days increasing to 300-400 tails at day 5.

Figure 412. Milk fish (*Chanos chanos*) fry, weakened by *Apiosoma.*

Epidemiology

Apiosoma feeds on organic particles and bacteria present in the water. They reproduce by binary fission and only use the fish host as an attachment substrate. Many can be free-living.

Pathophysiology

In large numbers, *Apiosoma* can cause mechanical blockage of respiratory surfaces on the gills, and particularly in small fry. *Apiosoma* do not invade or feed on the fish tissues but can act as a nidus for bacterial colonisation on the gills or skin. The source of these ciliates is possibly through the use of live feed collected from ponds that also contain *Apiosoma.* Changes in salinity may reduce the defences of the newly stocked fish thus allowing increased growth of these ciliates.

Diagnosis

Wet mount preparations of the skin and gills readily reveal the presence of ectocommensal protozoa. *Apiosoma* has a bell or vase-shaped body attaching to the fish surface by means of a holdfast organelle – the scopula. A thin layer of a sticky substance secreted by the scopula attaches the ciliate to the fish surface. The presence of oral cilia with no equatorial cilia identifies it as an *Apiosoma* (Figs. 413-415).

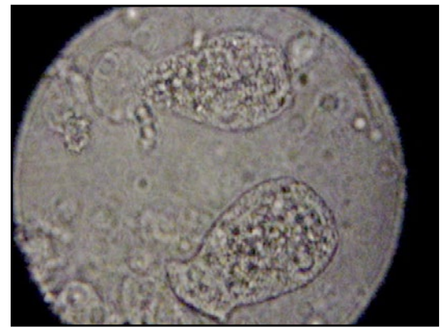


Figure 413. Wet mount of gills showing bell-shaped ectocommensal protozoa – *Apiosoma* sp.

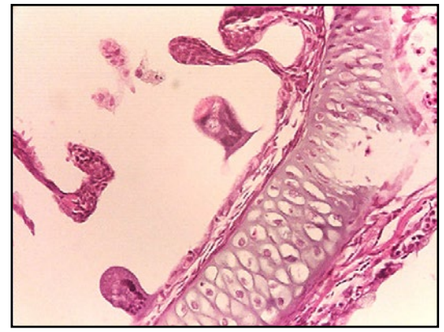


Figure 414. *Apiosoma* on gill surface; note that attachment is not permanent, but the ciliates are quite mobile.

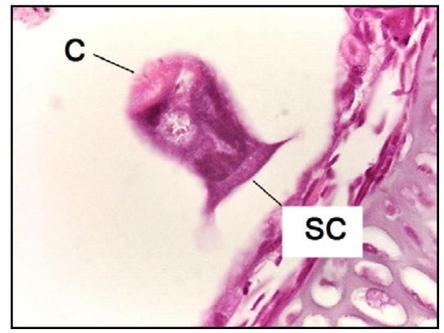


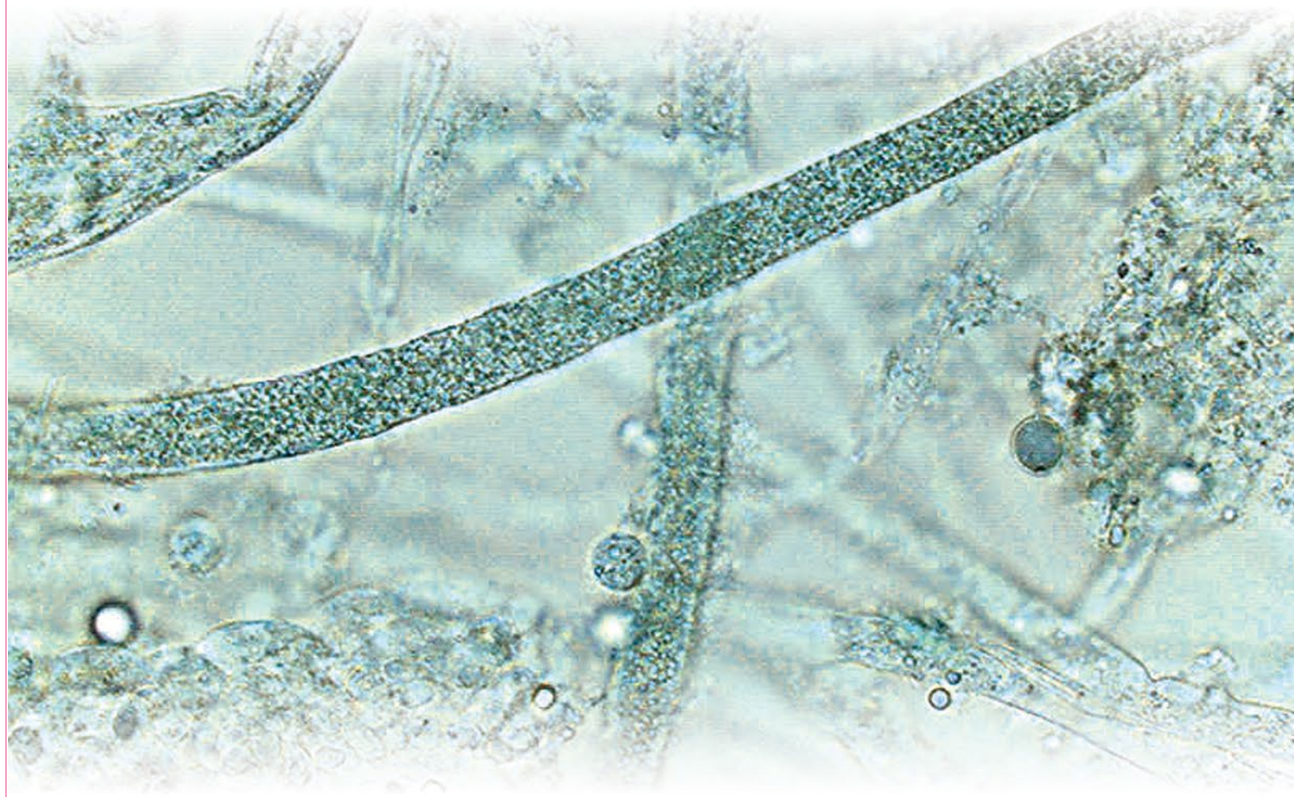
Figure 415. *Apiosoma* with oral cilia (C) and scopula (SC) hold-fast organ.

Control and Prevention

Management of apiosomiasis includes:

- Improving the water quality by filtration to remove organic debris
- Formalin bath at 10 ppm once
- Providing constant aeration
- Maintaining salinity until disease is under control
- Initial feeding using hatched brine shrimp, which is clean, to avoid exposure to local ectocommensal ciliates

PART 6 – FUNGAL & CHLAMYDIAL DISEASES



PART 6 –
FUNGAL & CHLAMYDIAL DISEASES

Microsporidiosis - Glugeosis

Farm History

Microsporidians are commonly detected in marine fish. They have recently been reclassified taxonomically as fungi. They are not noted to be associated with significant fish mortalities; however impacts on fish growth are possible and the issue of food safety concerns is relevant to the discussion. Species affected are grouper - *Epinephelus areolatus, E. coioides, E. fuscoguttatus*, snapper - *Lutjanus russelli* - and yellow-finned seabream - *Pagrus* sp..

Clinical Signs

Infected fish may display swelling of the abdomen, spore containing nodules or xenomas formation be extensive; otherwise there may be few gross external signs. On necropsy, the most obvious lesions are melanised or blackened xenomas in or on the visceral organs of the abdomen, particularly the peritoneal lining of the liver, and intestines. Some xenomas may be localised in the liver parenchyma. The number and size of these xenomas are variable but there can be considerable space occupying lesions. The cut surface of the xenoma produces a white and milky fluid full of spores. The xenomas have not been found in the muscle fillet of fish (Figs. 416-422).

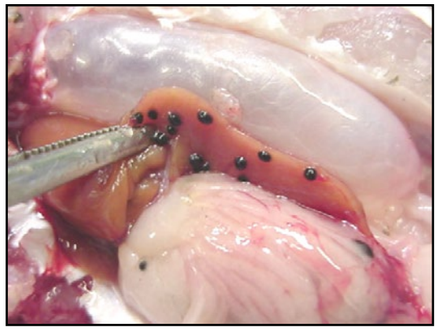


Figure 416. Liver xenomas, early glugeosis; brown spotted grouper (*Epinephelus areolatus*).

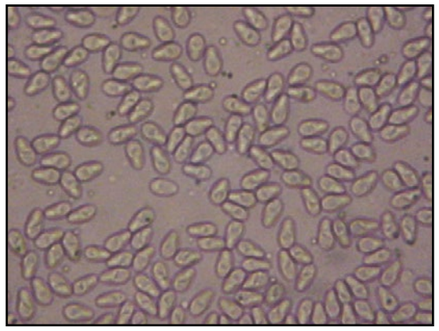


Figure 417. *Glugea* spores, wet mount; brown spotted grouper (*Epinephelus areolatus*).

Figure 418. Large group of melanised and 'metallic' looking xenomas, advanced stage glugeosis; brown spotted grouper (*Epinephelus areolatus*).

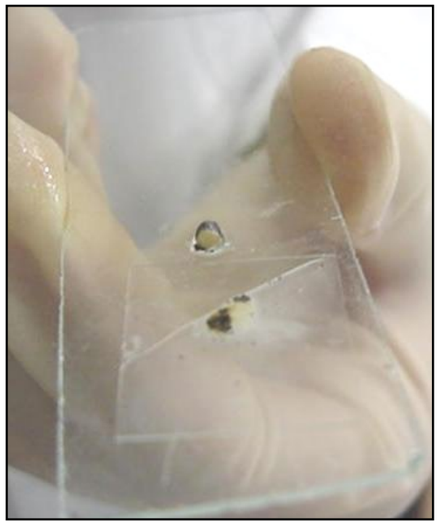


Figure 419. Cut surface of xenoma showing white and milky contents; only the capsule is melanised and black.

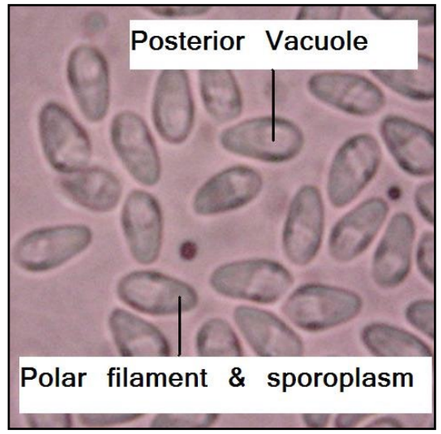


Figure 420. Spores of *Glugea* sp..

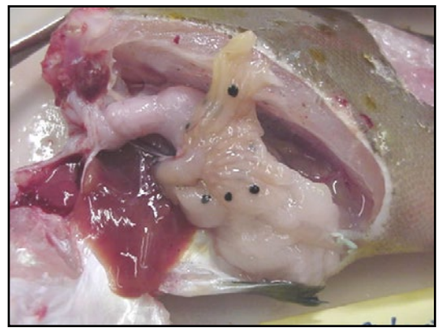


Figure 421. Peritoneal xenomas adjacent to pyloric caeca, early glugeosis; brown spotted grouper (*Epinephelus areolatus*).

Figure 422. Advancing glugeosis with more and larger xenomas spreading across the peritoneal membranes; brown spotted grouper (*Epinephelus areolatus*).

Epidemiology

The life cycle of microsporidians is possibly direct and involves the development of spores within the definitive fish host. There is some evidence of host preference: *Epinephelus areolatus* appears to have a higher prevalence of microsporidians in Hong Kong. However the same microsporidian from *Epinephelus areolatus* appears to infect other fish species on the farm. Cross-infection is dependent on the release of spores from infected fish when they die, probably from other causes. It is possible that the practice of trash fish feeding could promote the spread of microsporidians in mariculture.

Pathophysiology

Microsporidians form an enlarging xenoma through the multiplication of spores in host tissue. This xenoma is well encapsulated with a thick wall and is surrounded by host melanomacrophages producing melanin. The xenoma consists of spore germinating centres around the outer edge with spores being 'pushed' into the centre of the xenoma. Merogony and sporogony take place within the xenoma. Apart from the melanisation, there is little evidence of any systemic inflammatory response or systemic tissue damage. Large xenomas displace vital organs in the abdominal cavity and can represent an energy or metabolic cost to the host. Some level of immunosuppression may be involved although this has yet to be demonstrated for this species of microsporidian (Figs. 423-426).

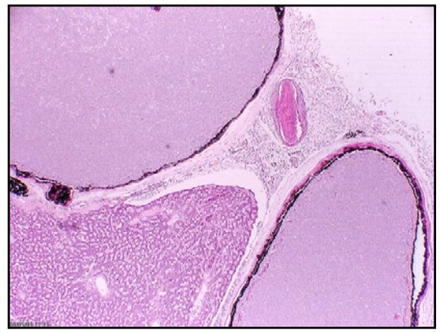


Figure 423. (H&E) Xenomas of the peritoneal lining of the liver; brown spotted grouper (*Epinephelus areolatus*).

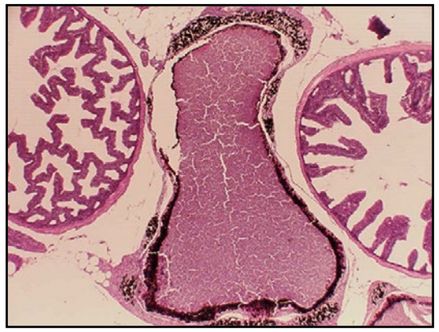


Figure 424. (H&E) Xenoma wedged between two sections of pyloric caeca; brown spotted grouper (*Epinephelus areolatus*)

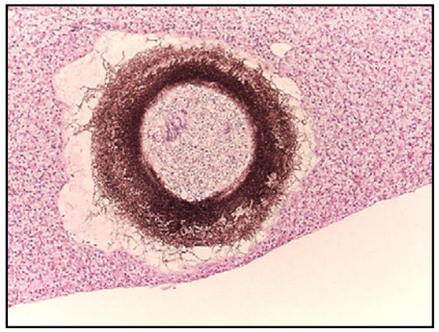


Figure 425. (H&E) Xenoma in the liver parenchyma; brown spotted grouper (*Epinephelus areolatus*).

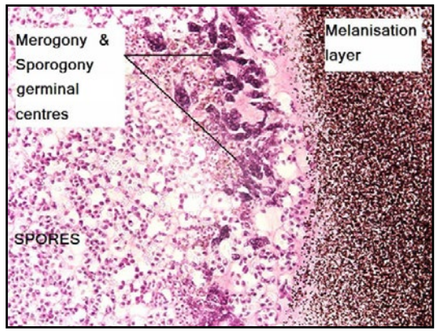


Figure 426. (H&E) Section of xenoma showing ultrastructure; brown spotted grouper (*Epinephelus areolatus*).

Diagnosis

The morphology of the microsporidian spore in Hong Kong marine fish is ovoid. It contains a single polar capsule and sporoplasm, and posterior vacuole. The formation of xenomas indicates that the *Glugea* sp. is involved. Reported marine microsporidians that are xenoma forming include *Glugea stephani*, and are reported to involve the connective tissue of the gastrointestinal tract (Woo, 1995; Noga, 1996). Wet mount examinations of cut xenomas or squash preparations readily demonstrate the presence of typical spores. Histology of the xenomas reveals the encapsulated and germinal structure. Giemsa staining highlights the spore structure. Species identification requires molecular and genetic sequencing methods by a specialist aquatic animal parasitologist.

Control and Prevention

There are some reports of experimental chemotherapy for microsporidian infections using toltrazuri and fumagillin (Langdon, 1992; Noga, 2010). Toltrazuril is ineffective against spore stages although there is some efficacy as a bath treatment against vegetative stages of microsporidians. Drug toxicity and efficacy issues with relapsing infections are associated with fumagillin. There are also microsporidian species resistant to these chemicals. Albendazole can treat *Loma salmonae* and quinine hydrocholride delays but does not prevent xenoma formation (Speare *et al.*, 1999).

Thus the following husbandry measures may reduce the spread of the disease:

- Avoidance of trash fish feeding
- Daily and prompt removal of dead fish
- Stocking of fish that do not show evidence of infection

Microsporidiosis – Neon Tetra Disease

Farm History

Pleistophora hyphessobryconis has been detected in neon tetra (*Paracheirodon innesi*). As with glugeosis, it is not reported by farms to cause significant mortalities, but is potentially a problem in fish intended for export. Therefore health testing and exclusion of infected batches are necessary for quality assurance. Microsporidians have recently been reclassified taxonomically as fungi.

Clinical Signs

Infected fish may show no external signs where the parasite load is low. In heavy infestations, whitening or opacity of the skeletal muscle, together with emaciation of fish may be observed. Deformities have been reported in the literature (Noga, 1996). Colour loss, particularly of the red banding along the caudal peduncle may indicate infested fish, but it is not pathognomonic (Fig. 427).

Figure 427. Neon tetra (*Paracheirodon innesi*) infested with *Pleistophora hyphessobryconis*; note the muscle emaciation (arrows) in some fish, which also show colour fading of the red band.

Epidemiology

Neon tetra disease is not restricted to *Paracheirodon innesi*, and can infest up to 16 species of freshwater fish including other tetras, goldfish, zebra danio and lineatus (Woo, 1995; Noga, 1996). Nevertheless, in Hong Kong it has only been detected in neon tetras during health testing from 1999 – 2003. As with other microsporidians, *Pleistophora hyphessobryconis* forms spores, which can infect susceptible fish in a possibly direct life cycle. Spores may be released into the environment through muscle damage or through cannibalism of dead fish by co-habiting fish, e.g., in a community tank.

Pathophysiology

Pleistophora hyphessobryconis does not develop a xenoma structure but forms sporophorous vesicles (SPVs). Localised myositis may indicate rupture of mature SPVs, releasing spores (Woo, 1995) while intact SPVs elicit little if any host inflammation. Enlargement of the SPVs replaces normal muscle fibres, leading to myodegeneration and opacity. SPVs may also be found in other tissues apart from muscle, e.g., gills, spinal cord, thymus and intestinal serosa. In zebra fish (*Danio rerio*), infection by *P. hyphessobryconis* results in numerous spores being phagocytosed by macrophages in the visceral organs including the ovaries (Sanders *et al.*, 2010). Granuloma response may be noted in these non-muscular sites of infestation (Figs. 428-435).

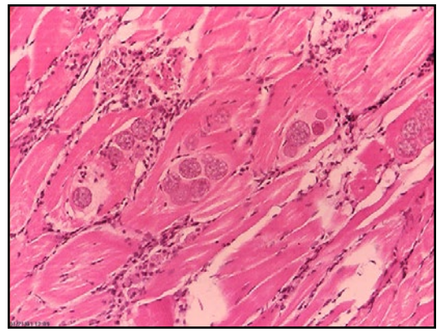


Figure 428. Sporophorous vesicles in muscle; neon tetra (*Paracheirodon innesi*) disease.

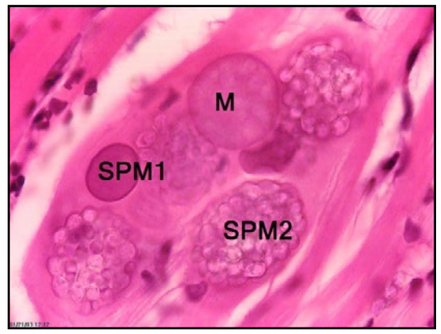


Figure 429. Sporophorous vesicle within the sarcoplasm of muscle fibre; meront (M), developing sporogonial plasmodium (SPM1) and mature spores (SPM2) within sporogonial plasmodium; neon tetra (*Paracheirodon innesi*) disease.

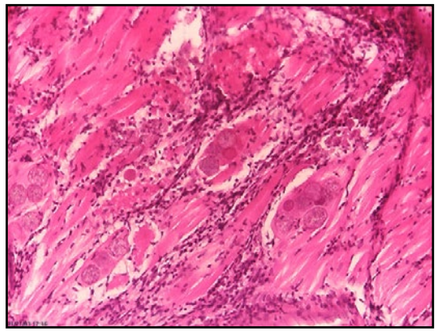


Figure 430. Lymphocytic myositis associated with neon tetra (*Paracheirodon innesi*) disease.

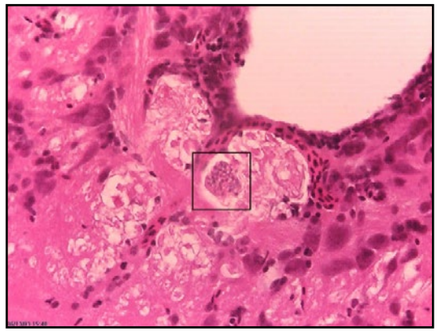


Figure 433. Neon tetra (*Paracheirodon innesi*) disease; sporophorous vesicle (box) in spinal cord.

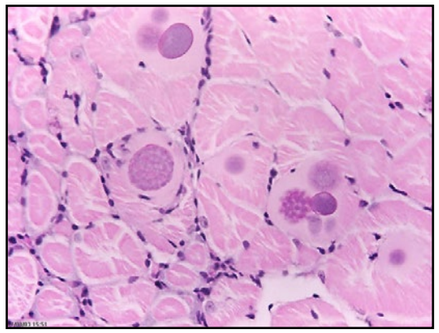


Figure 431. Note the lack of inflammatory response around the intact SPVs. Neon tetra (*Paracheirodon innesi*) disease.

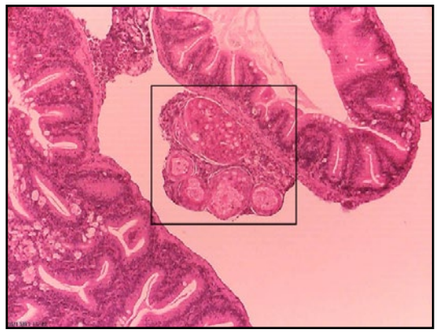


Figure 434. Granulomas with spores in serosa of intestine (box); neon tetra (*Paracheirodon innesi*) disease.

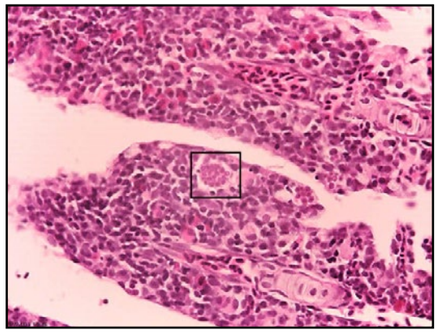


Figure 432. Gill hyperplasia in neon tetra (*Paracheirodon innesi*) disease; note sporophorous vesicle (box).

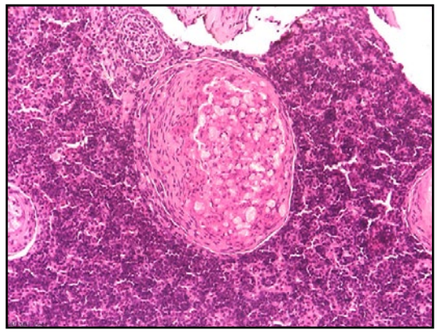


Figure 435. Granulomas with spores in thymus; neon tetra (*Paracheirodon innesi*) disease.

Diagnosis

If a fish shows whitish opacity of the muscle, a squash-wet mount preparation will reveal spores. *Pleistophora hyphessobryconis* spores are ovoid to pyriform with an anterior polar filament and sporoplasm, and a larger posterior vacuole. Histological sections of affected muscle will reveal the SPVs as foreign inclusions within the sarcoplasm of muscle fibres. Giemsa staining highlights the SPVs and spore structures (Fig. 436).

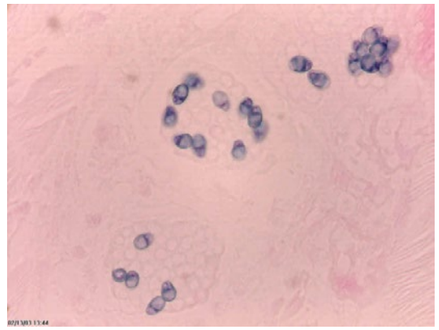


Figure 436. Giemsa stain of SPV showing spores of *Pleistophora hyphessobryconis.*

Control and Prevention

There are no reported effective treatments for neon tetra disease. Control depends on:

- Eliminating infected batches of fish from the farm
- Removing dead fish to discourage cannibalism
- Disinfecting tanks to reduce the environmental survival of spores, which may be up to 1 year (Noga, 1996)

Saprolegniasis

Farm History

Fungal disease due to *Saprolegnia* occurs in freshwater environments and is generally a reflection of suboptimal husbandry and pond hygiene. Species affected include mullet and milk fish (*Chanos chanos*) (Fig. 438) grown in freshwater ponds.

Clinical Signs

White cotton wool-like growths over the body of infected fish are commonly observed. This causes erosion of skin fins and tail (Fig. 437a) or scale loss (Fig. 437b).

Figure 437a. Saprolegniasis causing tail erosion in grey mullet (Mugilidae) fry.

Figure 437b. Milk fish (*Chanos chanos*) with scale loss due to *Saprolegnia.*

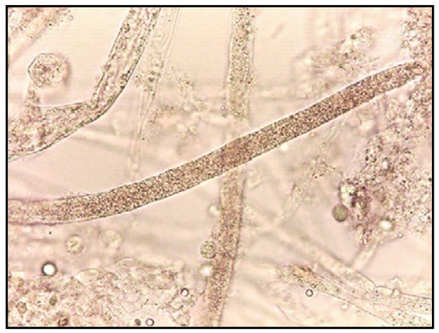


Figure 438. *Saprolegnia* hyphae; wet mount of milk fish (*Chanos chanos*) skin.

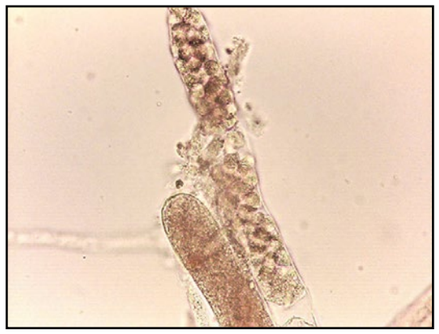


Figure 439. *Saprolegnia* zoosporangium.

Epidemiology

Saprolegniasis is associated with low water temperatures, which depress the immune responses of fish. Disease occurs following body abrasions on fish. An organically enriched environment favours the colonisation of fungal zoospores (Figs. 439, 441-442) and fungal hyphae development over damaged areas of the integument (Fig. 440).

Pathophysiology

Fungal hyphae invade the skin and muscle layers leading to osmotic imbalance. Secondary bacterial infection due to *Aeromonas hydrophila* is the likely sequelae. Fish mortalities are due to the osmoregulatory stress and bacterial infection.

Diagnosis

Wet mounts of the cotton-wool growths are used to identify thin, non-septate and un-branched hyphae with terminal zoosporangium indicative of oomycetes. In histology, silver stains are used to detect fungal hyphae in the necrotic areas of skin or muscle.

Control and Prevention

For effective control these measures are necessary:

- Remove all moribund fish
- Salt bath at 5 ppt continuously
- May need to treat for secondary bacterial infections
- Reduce stocking density
- Avoid body abrasions to fish such as in overcrowding and rough handling (net rash)
- Clean up the organic wastes in the pond with drying and liming periodically between fish crops

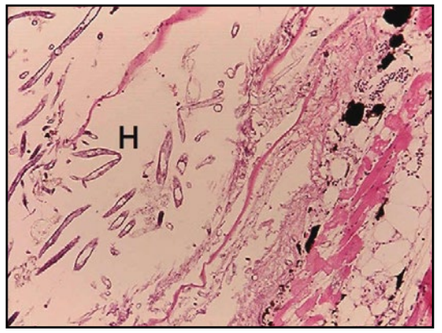


Figure 440. *Saprolegnia* fungal dermatopathy; note the hyphal structures (H).

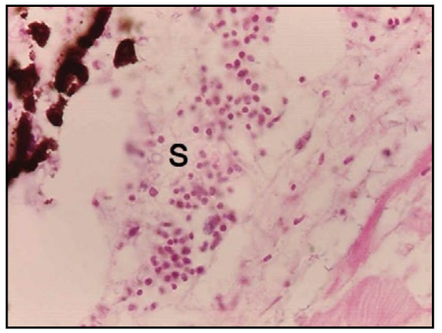


Figure 441. *Saprolegnia* spores (S) and secondary bacterial infection of damaged skin (H&E).

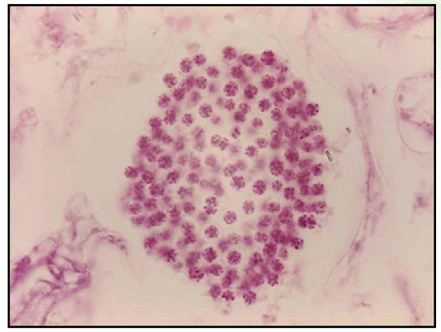


Figure 442. *Saprolegnia* spores in milk fish (*Chanos chanos*) skin (H&E).

Epitheliocystic

Farm History

Infections of epitheliocystis are generally subclinical in marine and freshwater fish species. As a group, they are not particularly pathogenic but it is necessary to distinguish epitheliocystis from other conditions such as lymphocystis and rickettsial infections. Epitheliocystis infects a wide variety of fish species including goldfish, koi carp, discus, red snapper, pompano, giant grouper and green grouper.

Clinical Signs

While epitheliocystis is localised in the gill lamellae and may be associated with lamellar hyperplasia (Figs. 443, 446) infected fish seldom display clinical effects as most cases have either single lesions or light infections. Heavy infections on the gills may be visible grossly as whitish patches.

Epidemiology

Little is known about the route of transmission of the epitheliocystis organism, although it is thought to be an infectious chlamydial organism (Noga, 1996). In some species , e.g., seabream, amberjack and mullet, and in heavy infections, it can cause significant mortalities (Brown, 1993; Noga, 1996).

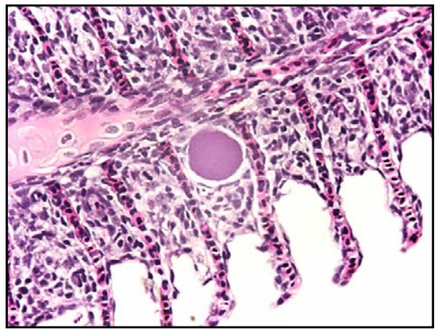


Figure 443. Goldfish (*Carassius auratus*) gills with an epitheliocystis lesion; note associated lamellar hyperplasia (although whether this is specifically caused by the lesion is not certain).

Pathophysiology

Epitheliocystis infects gill lamellar epithelial cells leading to individual cell hypertrophy. There may be variable degrees of lamellar hyperplasia around the lesion or none at all, leaving the lesion as a benign structure (Figs. 443-447).

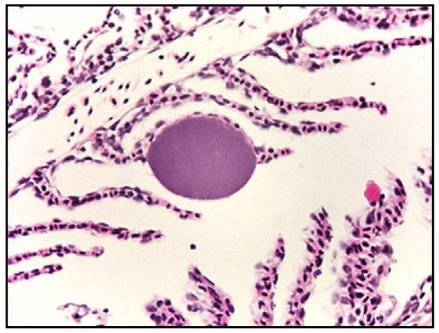


Figure 444. Discus (*Symphysodon* sp.) with single epitheliocystis; note the lack of host response and hypertrophy of infected lamella.

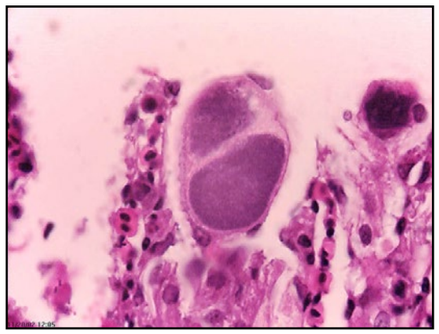


Figure 445. Red snapper (*Lutjanus malabaricus*) with gill epitheliocystis; note the 'dividing' lesion on the tip of the gill lamella.

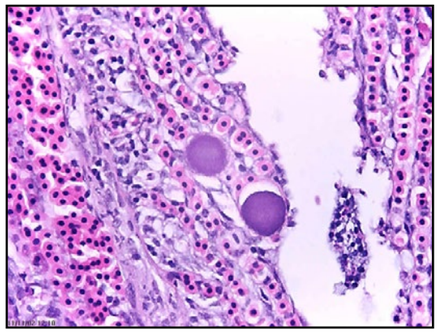


Figure 446. Koi carp (*Cyprinus carpio*) with gill lamellar hyperplasia and fusion associated with two epitheliocystis lesions.

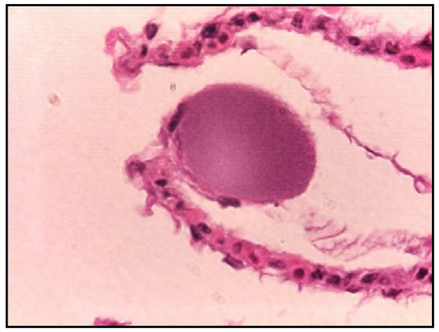


Figure 447. Red snapper (*Lutjanus malabaricus*) with gill epitheliocystis appearing to detach from the lamella.

Diagnosis

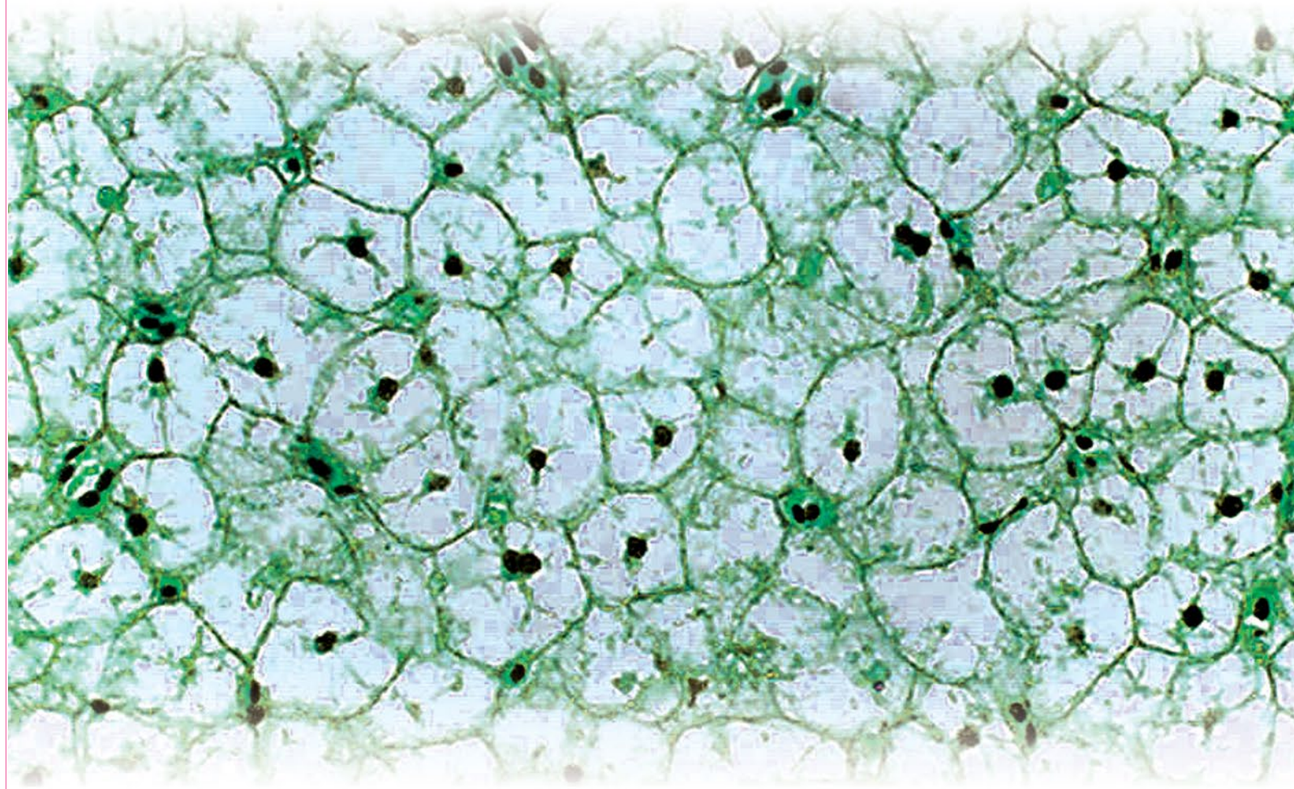
Histologically, the epitheliocystis lesion has these features:

- A thin rim of cell membrane surrounding an ovoid cyst-like structure in the gills
- Basophilic or purple staining material that is finely granular and filling the cyst
- Lesion found in the tip of lamellae or embedded in the basal interlamellar epithelium

Control and Prevention

Due to its low pathogenicity in local fish species, no specific control measures are required except where infections are heavy. In such cases, quarantine and disinfection of infected fish batches are applicable. Oxytetracycline has been reported to be effective against epitheliocystis in fish and ultraviolet irradiation of the water supply controlled outbreaks in amberjack and leopard coral grouper (Noga, 2010).

PART 7 – NON-INFECTIOUS DISORDERS



PART 7 – NON-INFECTIOUS DISORDERS

Non-infectious disorders by definition imply that parasites, bacteria, viruses and other infective pathogens are not primarily involved in the development of a disorder, abnormality or disease that results in measurable or observable morbidity and mortality in a batch or population of fish. However it is common to observe that stressor effects from non-infectious factors increase the probability of infectious disease expression. Therefore a discussion of non-infectious disorders in fish will include reference to the risks of infectious disease development where appropriate.

Oxygen Depletion

Farm History

Oxygen depletion or hypoxia is defined as levels of dissolved oxygen (DO) below the species requirement. 5 mg/L (ppm) has been set as a general level of DO to maintain fish health (Brown, 1993; Stoskopf, 1993; Australian and New Zealand guidelines, 2000), although it is recognised that fish species will have DO requirements lower or greater than 5 ppm. Cold-water species tolerate 4-5 mg/L indefinitely and warm water species survive for long periods at 2-3 mg/L (Stoskopf, 1993). Hatchery fish may require greater than 5 mg/L (Brown, 1993).

Clinical Signs

Affected fish are reported to surface or hang near water inlets gasping for air with rapid gill cover movements and mouth breathing. Alternatively fish may become very lethargic, cease feeding or die suddenly with few clinical signs. The gills are the primary organ to examine with pale discolouration indicating anaemia in the fish, or excess mucus and sediment both conditions of which would render fish susceptible to hypoxia. Fish often display widely extended mouths and gill covers if hypoxia is severe enough to kill them (Fig. 448).

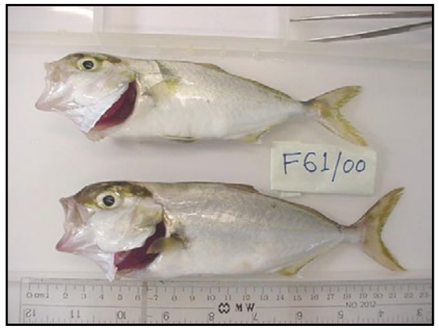


Figure 448. Greater amberjack (*Seriola dumerili*) with hyper extended mouths and gill covers; gill necrosis causes hypoxia.

Epidemiology

Low DO events may occur in mariculture due to:

- High summer temperatures.
- Water near the bottom of the seabed where anoxic organic sediments occur.
- Low tide and poor current flushing.
- After heavy feeding and in cages overstocked with fish.
- Red tide with any associated dinoflagellate die-offs.

Low DO events may occur in pond culture due to:

- Overstocking of ponds.
- Inadequate aeration or water exchange.
- Algal blooms and die-offs.
- Heavy rains and organic or sediment rich run-off contaminating the water supply.

Low DO events may occur in ornamental fish trading due to:

- Delayed airfreight transportation resulting in dead fish in fish bags.
- Overstocking of fish bags.
- Excessive excrement from fish not fasted or which have not undergone emptying of gut before bagging.

Pathophysiology

One of three pathogenic pathways leading to fish hypoxia is possible.

- Direct hypoxia – this occurs when the oxygen carrying capacity of the water is reduced and not resupplied due to either excessive consumption by biological (Biological Oxygen Demand - BOD) or chemical (Chemical Oxygen Demand – COD) processes in the water body. DO of 1 ppm or less is directly lethal to most fish (Noga, 1996; Stickney, 2000,).
- Indirect hypoxia – this occurs when there is a sudden increase in metabolic oxygen demand by fish for example after feeding and at higher water temperatures.
- Respiratory compromise induced hypoxia – this occurs when fish have severe gill disease, anaemia or are exposed to toxins that compromise oxygen exchange such as nitrite poisoning.

Diagnosis

Measurement of DO levels at the time of the hypoxic event and evidence of affected fish in the water provides a definitive diagnosis, but causal diagnosis requires the routine necropsy and histopathology of affected fish as well as assessing the recent history of the farm to identify risk factors. Measurement of the BOD or COD may provide an indication of DO upward or downward trending. High BOD or COD levels promote DO depletion.

Control and Prevention

Being aware of the risk factors in the environment of the fish culture system, DO measurement and monitoring, together with keen observation of fish are critical to prevent hypoxia. Examples of good DO husbandry include:

- Avoidance of overstocking.
- Availability and use of aeration.
- Avoidance of feeding during reduced DO readings.
- Control of gill disease due to parasites or bacteria.
- Availability and use of water exchange.
- Heeding red tide warnings and moving fish rafts to safe zones.

Endosulphan Toxicity

Farm History

This case of fish kill and toxicity reportedly began when a shop owner turned his back for 25 minutes from minding a tank full of marine fish (grouper and pompano) and returned to find 35 dead and two live fish (Fig. 449). The water of the aquarium was reported to be 'dirty and 2 plastic bags were found inside the aquarium' when the specimens were collected.

Clinical Signs

Tiger grouper and pompano were submitted for necropsy with these findings:

- Tiger grouper: The skin had excessive mucus. The liver was very pale with congested areas. The spleen was enlarged (Fig. 450). The intestines had larval worms, which migrated outside the intestine and were present in the abdominal cavity. The omental fat contained Glugea parasitic spores.
- Pompano: The kidney was haemorrhagic and the gills were autolysed.

Figure 449. Pompano (*Trachinotus blochii*) and tiger grouper (*Epinephelus fuscoguttatus*).

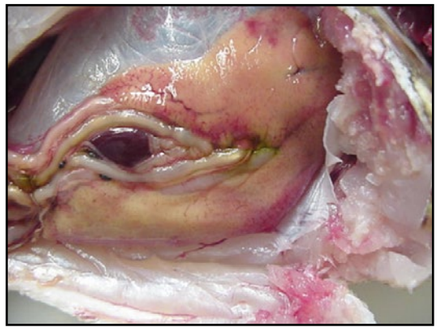


Figure 450. Tiger grouper (*Epinephelus fuscoguttatus*) liver – pale with areas of congestion; note the enlarged spleen.

Epidemiology

As water filtration equipment failure was ruled-out, the sequence of events was suggestive of malicious poisoning of the fish.

Water chemistry results with a dissolved oxygen of 6.8 mg/L ruled hypoxia as a cause, but the water was acidic for seawater (pH 6.75).

Pathophysiology and Diagnosis

Samples of whole fish showed excessive levels of an organochlorine pesticide:

- Endosulphan I - 640 µg/kg fish tissue
- Endosulphan II - 480 µg/kg fish tissue
- Endosulphan sulphate - 100 µg/kg fish tissue

This gives a total endosulphan residue in the fish of 1,220 µg/kg (ppb). This is three times the mean residue level of 0.4 mg/kg or 400 µg/kg detected in fish living in endosulphan-contaminated natural surface water (Gorbach and Knauf, 1971). Endosulphan is highly toxic to fish, and fish killed by endosulphan have been found to contain up to 1,000 µg/kg in the liver and muscle (Langdon, 1988). The highest exposure 96hour-LD_{50} reported for fish is 22 ppb or µg/L (EHC 40, 1984).

Histopathology consistent with toxicosis was:

- Tiger grouper: There was diffuse hepatocyte necrosis and heavy vacuolation with fatty degeneration (Fig. 451). Most of the hepatocytes had shrunken and pyknotic nuclei indicative of cell death. There was degeneration of the glomeruli with deposition of proteinaceous material, swelling of the glomerular tuft and reduction in the Bowman's space (Fig. 452).
- Pompano: There was heavy vacuolation and swelling of hepatocytes with multiple fat vacuoles. The cytoplasm of hepatocytes was more basophilic than normal.

Hepatopathies can be found in organochlorine toxicity (Couch, 1975).

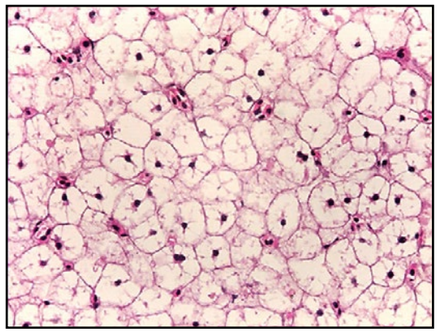


Figure 451. Tiger grouper (*Epinephelus fuscoguttatus*) – liver hepatocellular fatty necrosis.

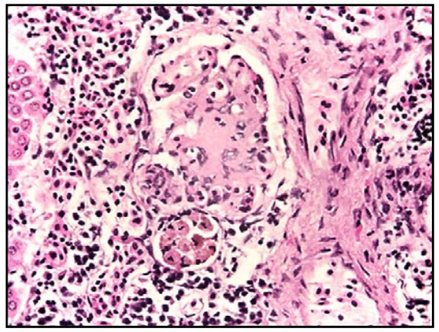


Figure 452. Tiger grouper (*Epinephelus fuscoguttatus*) – glomerular tuft degeneration; note the increased proteinaceous material in the tuft.

Chlorine Toxicity

Farm History

Inadvertent poisoning with chlorine can occur through the use of town water supplies that have not been dechlorinated before use. Similarly, exposure to elevated chlorine compounds in the culture environment can occur through the use of bleaching compounds for disinfection of farm utensils and failure to adequately rinse off residual chlorine. Two cases of chlorine toxicity are described here; case 1 was due to the use of town water, and in case 2, the source of chlorination was undetermined.

Clinical Signs

Case 1

Affected koi carp (*Cyprinius carpio*) gills had large areas of damage and necrosis with excessive mucus and green algae attached. Filaments were eroded and ragged in appearance. Fins and tail were reddened and inflamed.

Case 2

Yellow-finned seabream (*Sparus latus*) showed skin erythema at the base of the pectoral and pelvic fins, and tails. The gills had extensive blotches of haemorrhage with some green algae and sediment over the filaments. There was erythema of the mouth and the kidney was haemorrhagic and soft.

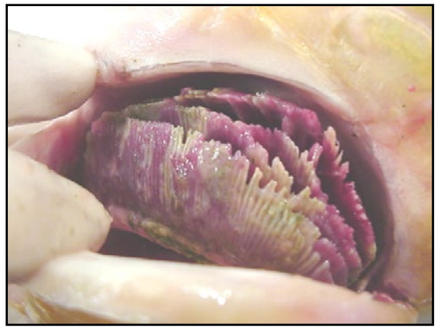


Figure 453. Koi carp (*Cyprinus carpio*) gill necrosis; chlorine exposure; note that Koi Herpesvirus (KHV) may also cause extensive gill necrosis.

Figure 454. Yellow-finned seabream (*Sparus latus*) with gill haemorrhages; chlorine exposure.

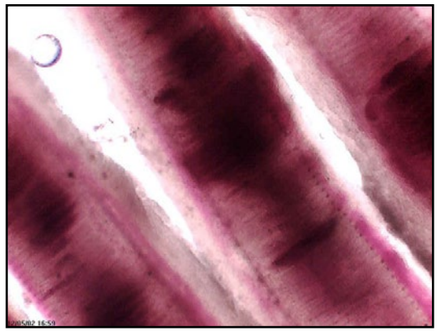


Figure 455. Wet mount of gills showing haemorrhages in yellow-finned seabream (*Sparus latus*); chlorine exposure.

Epidemiology

Case 1

Mortalities in affected koi carp (*Cyprinius carpio*) reached 50% in 1 week with 700 fish stocked in an urban ornamental fish pond.

Case 2

Mortalities in affected yellow-finned seabream (*Sparus latus*) were 15% of 900 fish in 2 months for pond-reared fish.

Pathophysiology

Chlorine causes gill degeneration and necrosis (Figs. 453, 457). There is, however, a wide range of potential clinical expressions, depending on the level of chlorine as well as the pH. Changes such as mainly anaemia where the levels are not enough to cause overt gill necrosis, but result in foci of cell damage and methemoglobinemia on red cells passing through the gills can occur, these being later removed by the spleen (Pers. Comm. Judith Handlinger). The severity of damage is concentration dependent. The toxicity of chlorine is pH dependent, being more toxic at lower pH. At pH 7.25, approximately 70% of total chlorine exists as the toxic HOCl. At pH 8, approximately 18% of total chlorine exists as the toxic HOCl. Chlorine acts as an oxidizing agent, at high levels affecting the gill tissue directly (Heath, 1995). The recommended safe long-term exposure level is total chlorine < 0.003 ppm (ANZECC, 2000). Total chlorine levels ≥0.1-0.4 ppm have been associated with fish kills (Langdon, 1988) for freshwater species and ≥0.128-0.250 ppm for marine species (ANZECC, 2000). Physiological effects include osmoregulatory and respiratory failure. Depending on the fish species, gill responses range from hyperplasia to branchitis and necrosis.

Case 1

Koi carp histology – gill with moderate lamellar hyperplasia (Fig. 456)

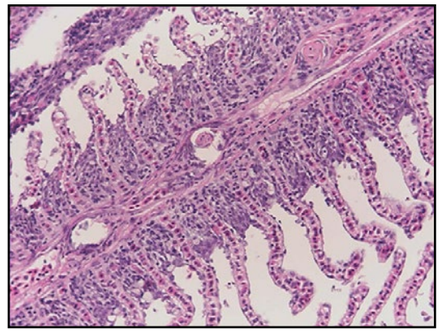


Figure 456. Koi carp will lamellar hyperplasia – no viral inclusions as in the Koi Herpes Virus (KHV); chlorine exposure.

Case 2

Yellow-finned seabream - extensive degeneration and necrosis with lamellar epithelial lifting and many eosinophilic granulocytes and polymorphs (neutrophils) in the gill filament representing severe branchitis (Figs. 458-459). Eosinophilic granulocytes are on the edges of the primary filament and in the interlamellar area. Polymorphs are located at the base of the lamellae. Macrophages migrate onto the lamellar epithelium.

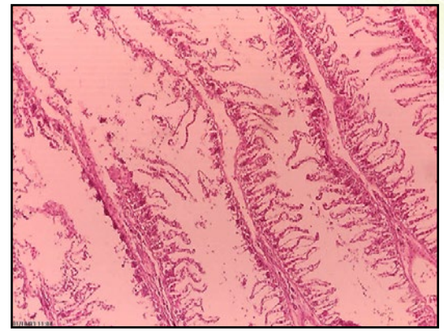


Figure 457. Gill necrosis; yellow-finned seabream (*Sparus latus*) with chlorine exposure.

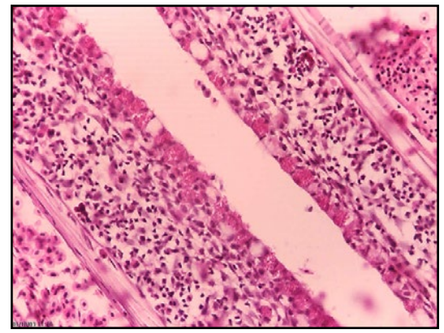


Figure 458. Branchitis with eosinophilic granulocytes; yellow-finned seabream (*Sparus latus*) with chlorine exposure.

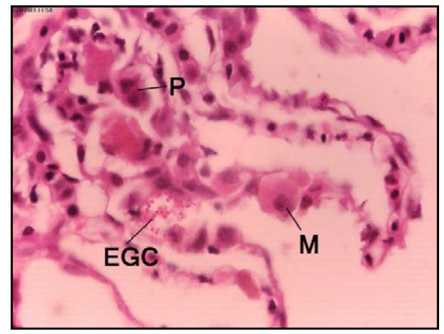


Figure 459. Branchitis with eosinophilic granulocytes (EGC), macrophages (M) and polymorphs (P); yellow-finned seabream (*Sparus latus*) with chlorine exposure.

Diagnosis

Diagnosis is dependent on:

- Analysis of total chlorine levels in culture water –
  - Case 1: chlorine 0.13 ppm and pH 7.18.
  - Case 2: chlorine 0.01 - 0.05 ppm and pH 7.28 – 8.14.
- Evidence of damage to gills or other tissues consistent with oxidative damage.
- Absence of primary infectious disease, e.g., viral, bacterial or parasite gill diseases.
- Evidence of source(s) of chlorine
  - It would appear that in comparing the two cases, the carp which were exposed to higher chlorine concentration had more severe and acute mortalities of fish while the yellow-finned seabream had lower and more chronic mortalities at lower chlorine concentrations.
  - The histopathology of the gills suggest that carp may be more tolerant than yellow-finned seabream to chlorine because hyperplastic changes in the carp gills were observed while only acute inflammatory and necrotic changes occurred in the yellow-finned seabream.

Control and Prevention

Options to reduce losses include:

- Dechlorination with sodium thiosulphate or water exchange using chlorine-free water.
- Increasing aeration.

Prevention is performed by avoiding exposure to residual chlorine from known sources and monitoring of chlorine levels in the culture water.

Microcystis Algal Toxicity

Farm History

Algal blooms can easily occur in pond fish culture where water exchange is limited and stocking density is high. Problems occur when the algal bloom dies off or if toxic species of algae become a major proportion of the algal mix. Differentiating between the two conditions is important with respect to management strategies.

Figure 460. Pond-based fish culture – a semi-closed system.

Figure 461. Heavy rains had muddied the water supply channel or stream.

Figure 462. Dead pompano (*Trachinotus blochii*) in the algae-filled pond water; note the deep green colour.

Figure 463. Fish washed up all along the pond edge; note the algal bloom in the water.

Clinical Signs

Acute mortality of greater than 90% in pompano fish occurred over 2 to 3 days (Figs. 460-463). Fish were noted to be swimming in circles and upside down (Fig. 464). They had swollen abdomens prior to death. Necropsy findings included:

- Gills were green staining but deep red in colour indicating blood congestion.
- Kidney was a deep red colour pointing to blood congestion.
- Liver was pale yellow and friable.

Figure 464. Live pompano (*Trachinotus blochii*) sampled; note the upside down posture and no evidence of gasping gill activity indicative of adequate oxygenation.

Figure 465. Pompano (*Trachinotus blochii*) with very congested gills.

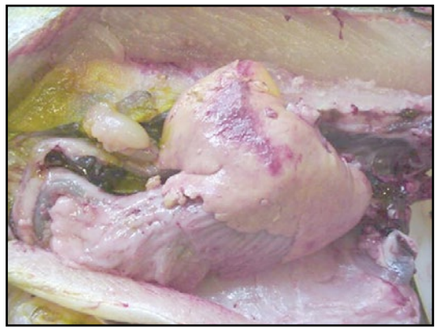


Figure 466. Pompano (*Trachinotus blochii*) liver friable and pale.

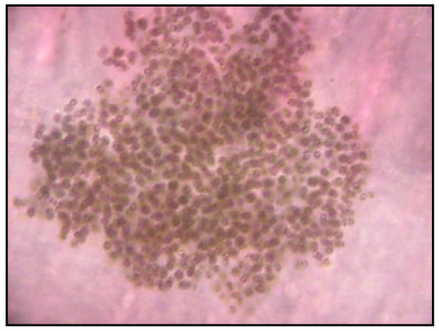


Figure 467. Pompano (*Trachinotus blochii*) gill wet mount showing a cluster of *Microcystis aeruginosa.*

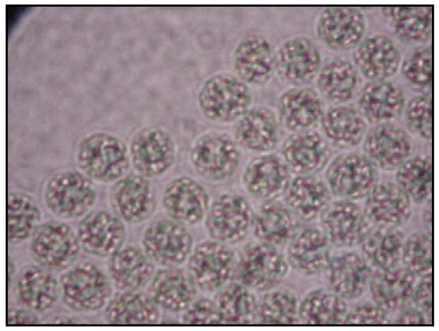


Figure 468. *Microcystis aeruginosa* unicellular and spherical algal cells.

Epidemiology

Fish started dying 2 days before the heavy rain which then muddied the river. This river was the water supply for the fish ponds. A lot of algae was found in the water of the affected fish pond during the fish kill (Figs. 460-463). As a result of the rains, clean water exchange to ameliorate the fish mortalities was not available.

Pathophysiology

Microcystis aeruginosa cyanobacteria or blue-green algae were detected in water samples; however it cannot be assumed to always produce the microcystin toxin (Janse *et al*., 2004). There are over 80 types of microcystins (MCs), depending on the amino acid profile. MC-LR, which contains leucine and arginine, is the most commonly found and studied microcystin toxin (Sigma-Aldrich Co., 2007). Other microcystins include MC-RR, MC-YR and MC-LA. MC-LR is a potent hepatotoxin that inhibits protein phosphatases that maintain the cytoskeleton of hepatocytes. Disruption of phosphate groups of the cytoskeleton proteins by MC-LR causes inward collapse and hepatocellular necrosis (Sigma-Aldrich Co., 2007). Microcystin has been observed to cause sublethal damage to the liver, kidney and intestine of fish at a dose of 0.6–0.9 µg microcystin-$LR_{equiv.}$ /kg body weight (Dietrich *et al.*, 2006).

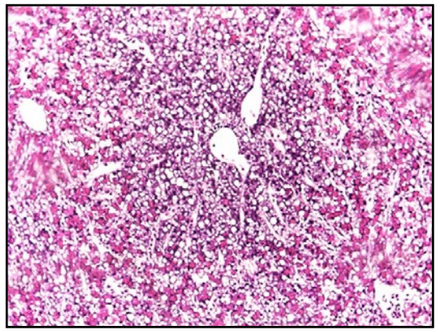


Figure 469. Pompano *(Trachinotus blochii)* liver; periportal hepatocellular necrosis and generalised eosinophilic degeneration of hepatocytes.

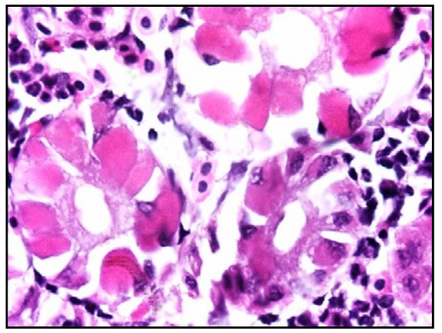


Figure 470. Renal tubular degeneration in pompano *(Trachinotus blochii)* with microcystin exposure.

Histopathology findings included:

- Liver –extensive hepatocyte degeneration and necrosis involving 80-90% of the liver section. Necrotic hepatocytes had dark staining and shrunken (pyknotic) nuclei with eosinophilic degeneration of the hepatocellular cytoplasm. These degenerate hepatocytes surrounded the necrotic hepatocytes, which in turn occurred around the hepatic central veins. Hepatocyte vacuolation containing fat globules was also present (Fig. 469).

- Kidney –necrosis and degeneration of renal tubular cells, which were swollen and filled with eosinophilic material similar to that of the liver. Approximately 30% to greater than 50% of renal tubules were affected. Affected tubular cells were detached from the basement membrane of the renal tubules. The renal tubular cell nucleus was marginated where the eosinophilic material had filled the entire cytoplasm. There was also necrotic change in the haematopoietic portions of the kidney (Fig. 470).

Diagnosis

The definitive diagnosis was based on:

- Evidence of algal bloom at the time of the fish kill; the pond water was deep green and contained a dominant algal species – *Microcystis aeruginosa*.

- Evidence of dissolved oxygen not depleted; DO in affected fish ponds were 7.76-13.95 ppm, indicative of an algal bloom producing oxygen and not dying-off.

- Evidence of liver and kidney damage not due to infectious or other causes.

- Evidence of microcystin toxins produced at toxic levels; the results of microcystin assay on water samples were:

  · MC-RR 1 mg/g dried biomass of algae

  · MC-YR 0.23 mg/g

  · MC-LR 0.57 mg/g

The acute toxic dose of MC-LR (the most toxic congener of microcystin) has been reported to be 5,700 µg/kg body weight of fish (Dietrich *et al.*, 2000). To calculate the risk quotient (RQ) or hazard quotient (HQ), where it is > 1, this would indicate an increased significant risk of toxicity.

MC-LR 0.57 mg/g = 570 mg/kg = 570 ppm

Toxic dose in trout = 5,700 µg/kg = 5.7 mg/kg = 5.7 ppm

Exposure dose/toxic dose (RQ,HQ) = 570/5.7 = 100

Pompano fish have been exposed to MC-LR concentrations in the water, which are 100 times the acute toxic tissue dose.

Control and Prevention

- Avoiding overstocking and overfeeding.

- Regular water exchange to reduce the build-up of excess organic wastes and monitoring of pond algae levels.

- Pond drying, sediment removal and liming between fish harvests.

- Critical to the management of fish health is the availability of clean water for rapid water exchange. This case illustrates the failure of this measure thereby resulting in serious fish losses.

Temperature Stress

Farm History

The effect of temperature on fish health cannot be understated. This is because temperature has a direct effect on the pathogen population and its pathogenicity while also influencing the physiological responses of the fish host, including aspects of its immune capability. Typically, sudden fluctuations in temperature trigger the onset of diseases. In general, downward temperature changes trigger fungal and parasitic outbreaks while upward changes favour bacterial conditions. If temperature changes fall outside the physiological optima for the species, the fish will die directly due to metabolic collapse even in the absence of significant pathogens (Fig. 471).

Case 1 - Cold stress

A fish kill occurred following cold weather some 7 days previously. The water temperature at the time of sampling was 19.8ºC. Fish were sluggish and not feeding. There was some change in the water colour with a rise in algal content but not of toxic species. Mortalities of 10% occurred in red snapper *(Lutjanus malabaricus)* and cobia *(Rachycentron canadum)* up to 100%. Some mortalities also occurred with pompano *(Trachinotus blochii)* and green grouper *(Epinephelus coioides)*. The pompano were noted to be jumping out of the water and a net covering had to be placed. Attempts were made to place the fish in heated tanks to increase the water temperature with freshwater baths and aeration but mortalities still continued. The brown spotted grouper had skin lesions but according to the farmer it seemed more resistant to the cold weather than the other species (Figs. 472-474).

Case 2 - Heat stress

Greater amberjack *(Seriola dumerili)* experienced up to 60% mortality in fish of 9-15 cm in length, which were stocked at 10,000 fish per raft. The water temperature (27 ºC) increased rapidly to 30 ºC in 10 days.

Figure 471. Cold wintery weather in Hong Kong mariculture site.

Clinical Signs

Case 1

Cobia were found dead with little external lesions and floating in the water. The median lethal temperature for cobia juveniles is 12.1 ºC with slow acclimatisation at 0.53 ºC per day (Atwood *et al.*, 2004) (Fig. 472).

Figure 472. 100% mortality in large adult cobia *(Rachycentrum canadum)* directly due to cold temperature stress.

Red snapper showed severe ulceration of the mouth and snout regions. The caudal, dorsal and pectoral fins were eroded and frayed. The liver looked pale and the spleen enlarged. The inner lining of the stomach was inflamed and haemorrhaged. The swim bladder was hyper inflated and the gall bladder enlarged. There was abundant fatty tissue in the abdomen.

Figure 473. Dead red snapper (*Lutjanus malabaricus*) due to cold stress.

In pompano, the gills were heavily infested with *Trichodina* parasites (Fig. 475) but surprisingly there was very little host reaction in the form of excessive mucus production although gill hyperplasia and fusion were present (Fig. 476). The presence of massive numbers of trichodinids explains the fact of the pompano jumping out of the water due to irritation from the feeding activities of the trichodinids. There was very little fatty tissue in the abdomen.

Figure 474. Netting placed over pompano (*Trachinotus blochii*) cage to prevent fish jumping out due to heavy gill irritation from trichodinids.

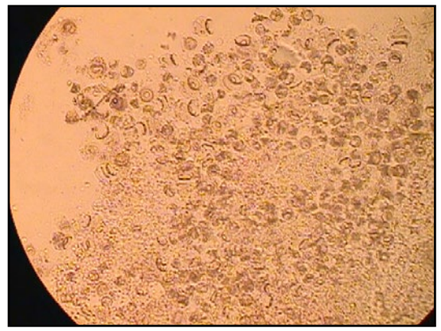


Figure 475. Large numbers of trichodina in pompano (*Trachinotus blochii*); cold stress.

Case 2

Greater amberjack (*Seriola dumerili*) showed darkening of the head, some body lesions and swam abnormally. The water chemistry parameters were:

Temperature - 30.6 ºC
DO – 4.8 ppm
Carbon dioxide – 23 ppm

Greater amberjack (*Seriola dumerili*) are a species of fish requiring cooler water temperatures of 18 – 27 ºC. The elevated water temperatures of 30.7 ºC may have a role to play in fish mortalities by reducing the availability of oxygen in the water. Furthermore, oxygen demand by the fish stocks is increased at higher temperatures such that relative oxygen deficiency can occur.

A high stocking density of 10,000 fish per raft can also induce crowding stress which can impair the exchange rate of water in the net pens causing a build-up of wastes such as carbon dioxide. Carbon dioxide impairs the absorption of oxygen by fish stocks.

Epidemiology

Winter water temperatures in Hong Kong mariculture sites can decrease from 20 ºC to 16.4 ºC in some fish culture zones. For fish species with temperature optima above 20 ºC, winter can be a significant low temperature stressor.

Summer water temperatures can exceed 30 ºC (average temperature of 27-29 ºC) and reported fish mortalities from stocking of fingerlings are usually higher. The associated risk factors are lower dissolved oxygen and higher bacterial counts in the water. Mortalities are more likely to be due to secondary bacterial disease such as vibriosis and oxygen depletion.

Pathophysiology

Low temperature stress can be caused by:

- a rapid reduction in the water temperature within the physiological temperature range for the fish species, leading to a reduction in the metabolic rate and delays in immune response times . This allows pathogens that prefer a low temperature range to cause opportunistic infections, particularly fungi and parasites on fish, which have skin or gill damage.
- a temperature reduction that falls outside the physiological temperature range for fish

species to the extent that the opportunity or ability of fish to acclimatise is inadequate; this can cause mortalities in the absence of pathogenic disease. This is associated with failure of osmoregulation and cardiovascular shock (Stoskopf, 1993).

High temperature stress can be caused by:

- a sudden increase in the water temperature within the physiological temperature optimal range for fish species; failure to acclimatise leads to fish mortalities by oxygen depletion and opportunistic infections.
- a temperature increase that exceeds the physiological temperature range for fish species; this causes mortalities through osmoregulatory failure.

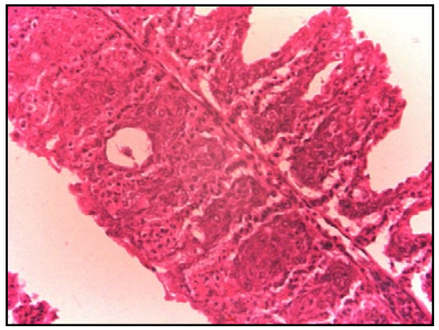


Figure 476. Gill lamellar hyperplasia and fusion in pompano (*Trachinotus blochii*); cold stress.

Diagnosis

Knowledge of the physiological temperature optima for the species concerned is required. In general a temperature change of greater than 1°C per hour is considered stress-inducing for fish (Noga, 1996), but this varies with fish species and salinity, life-stage and reproductive status of the fish sample. Temperature is a critical risk factor in most infectious diseases and this should be taken into account when investigating likely triggers for an epidemic.

Control and Prevention

Apart from slowly readjusting the water temperature of the culture system (often not possible), the following measures may alleviate losses:

- Treat opportunistic infections.
- Minimise handling.
- Adjust salinity,– e.g., a reduced salinity bath for marine fish to ameliorate osmoregulatory stress.
- Use supplementary aeration in high temperatures.
- Acclimatise new fish by floating the fish transport bags in the net pen until the temperature difference is significantly reduced (< 1°C) before releasing the fish.

Salinity Stress

Farm History

Salinity changes usually are associated with periods of heavy rainfall, which lowers salinity. It can also occur with the rapid transfer of fish from transport water to cage pens for newly arrived fish. Excessive treatment in freshwater for marine species may also cause salinity stress. Fish mortalities may follow about 1 week after the rain event and usually due to secondary infections.

Clinical Signs

Clinical signs are usually associated with secondary infections after rainfall events. In freshwater bath treatments that are too long, fish begin to turn dark and appear weak in the water column (Figs. 477-480).

Figure 477. Brown spotted grouper (*Epinephelus areolatus*) with gill necrosis due to vibriosis after heavy rainfalls and reduction in salinity to 24.2 ppt.

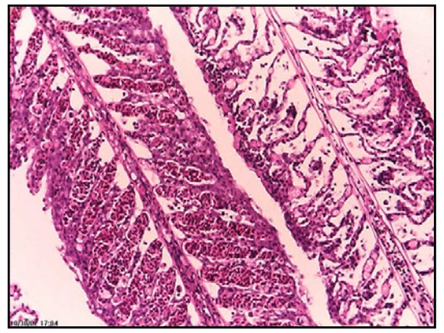


Figure 478. (H&E) Brown spotted grouper (*Epinephelus areolatus*) with gill necrosis and haemorrhage due to vibriosis after heavy rainfalls and reduction in salinity to 24.2 ppt.

Figure 479. Brown spotted grouper (*Epinephelus areolatus*) with ulcerative vibriosis following heavy rains.

Figure 480. Mullet (Mugilidae) fry exposed to 12 ppt when requirement is 30 ppt; secondary fungal infection (*Saprolegnia*) and mortalities; note the darkened fish, which is a general sign of stress.

Epidemiology

Marine fish usually require salinity in the range of 15-37 ppt, freshwater species tolerate up to 3 ppt and brackish species have a variable salinity range of 0.1-37 ppt (Langdon, 1988). As for temperature acclimatisation, acclimatisation to changing salinity can successfully occur provided that the change is gradual. As a rule of thumb, salinity should not vary greater than 1 ppt/hour or > 10 ppt in a few hours for estuarine fish (Noga, 1996).

Pathophysiology

Osmoregulation in fish is dependent on normal gill and kidney function, although the gills play a major role. Sudden decreases in salinity for marine species can result in net over hydration with resultant changes in serum electrolytes and osmolality. If there is existing gill damage, osmoregulatory stress is more likely. This may allow secondary infections to occur.

Diagnosis

Measurement of reduced salinities in a marine culture site following rainfall usually indicates a large inflow of freshwater to dilute the marine waters. It is important to note that various pollutants and organics may be included in the rain-run-off and that fish mortalities may not be solely due to salinity issues.

Control and Prevention

Where possible fish should be exposed to gradual changes in salinity. Management of secondary infections is important.

Swim Bladder Mal-inflation

Farm History

Hatchery fish fry are susceptible to problems with swim bladder inflation. Failure to inflate can result in problems with buoyancy and normal swimming. This then contributes to inability to catch feed. Mortalities are the result of starvation as the yolk sac nutrient reserves are consumed.

Sweet-lip fry were hatched 14 days and reared in tanks. Whirling swimming was observed and mortalities commenced.

Figure 481. Culture tank with sweet-lip (*Lethrinus Miniatus*) fry.

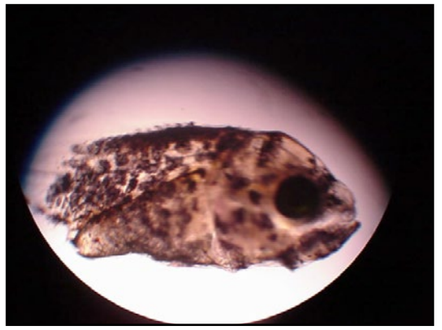


Figure 482. Wet mount; sweet-lip (*Lethrinus Miniatus*) fry with distended anal portion.

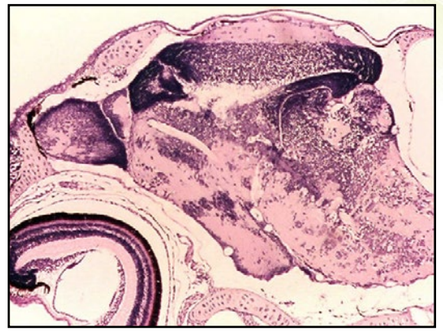


Figure 483. (H&E) Normal brain and retina of sweet-lip (*Lethrinus Miniatus)* fry.

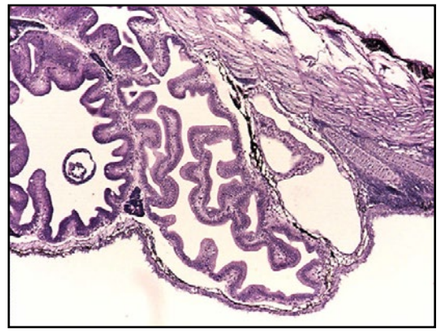


Figure 484. (H&E) Distended and protruding gut of sweet-lip (*Lethrinus Miniatus*) fry.

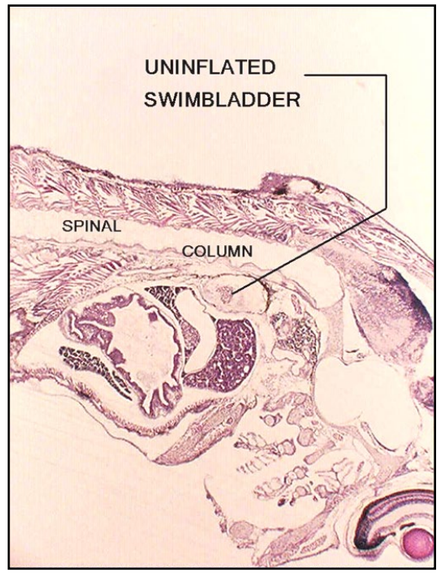


Figure 485. (H&E) No swim bladder inflation in sweet-lip (*Lethrinus Miniatus*) fry.

Epidemiology

Possible reasons why initial swim bladder inflation fails in marine fry (based on studies in *Pagrus Major*) are:

- Oily contamination of the water surface or floating debris, such that fry cannot break through the water-air interface to gulp air.
- Excessive turbulence, e.g., from aeration or water pumping currents in the rearing tank which interferes with fry swimming up.
- Dietary deficiency of n-3 highly unsaturated fatty acids (n-3 HUFA) either in the rotifers or in the broodstock diet; it was observed that these fry deficient in n-3 HUFA resulted in low swimming activity, a lack of endurance and improper reflex responses. Consequently they could not swim up and inflate the swim bladder. In studies with striped trumpeter *(Latris forsteri)* gut lesions occurred with similar HUFA deficiencies and may explain the appearance of expanded guts (Pers. Comm. Judith Handlinger).
- Exposure to teratogenic chemicals may impair the proper development of the primordial swim-bladder and adversely impact on inflation in the fish fry (Perlberg *et al.*, 2008).

Pathophysiology

There was no inflation of the swim bladder in the sweet-lip fry, which could have produced the swimming abnormalities. Mortalities were probably related to the inability to catch food normally. The marked distension of the gut caused the caudal abdominal protrusion but the gut in most specimens was empty of rotifer food. Only one of the fry sectioned actually had a rotifer ingested. This distension of the gut may be filled with fluid (perhaps seawater) as fish attempted to catch feed but perhaps only managed to swallow water. This over-filling of gut with seawater may also contribute to the swimming abnormality. Also these fry did not appear to eat properly as the gall bladder was enlarged with bile and the liver was lacking in lipid vacuoles.

Diagnosis

Examination of the fry under whole live wet mount before euthanasia and fixation for histology is necessary to visualise the gut distension and the lack of swim bladder inflation. Observation of the swim pattern of the fry will enable evaluation of abnormalities with buoyancy and stability of the fish in the water column. The differential diagnosis for abnormal swimming in fish fry includes nodavirus infection; therefore examination of the retina, brain and spinal cord for vacuolative changes is important (Figs. 236-239).

Control and Prevention

Reducing risk factors for swim bladder mal-inflation includes:

- Ensuring that there is no oily contamination of the water.
- Minimising excessive aeration and water turbulence.
- Dietary supplements of HUFAs such as docosahexaenoic acid (DHA) and eicosapentaenoic fatty acid (EPA) in the broodstock diet or live feeds such as rotifers or artemia (Bromage and Roberts, 1995).

Fatty Liver Syndrome

Farm History

Fatty liver syndrome is a common finding in cultured fish primarily due to the high levels of nutrition farmers apply in order to achieve high growth rates. Health problems occur when the species of fish does not tolerate the level of fat included in the diet.

Clinical Signs

Fish suffering from fatty liver syndrome show a pale yellow (Fig. 487) to almost white liver (Fig. 489), full of lipids. However, some fish livers appear brown (Fig. 486) and normal looking but also contain high levels of lipid. Fish may be anaemic. It is important to differentiate fish species that normally use the liver to store large amounts of fat but are not sick with fatty liver syndrome. This is especially so with cultured fish which are offered regular feeding enabling these to store lipids while feral fish that have to forage for feed are less likely to have excessive fat in the liver.

Figure 486. Normal looking liver – red/brown; Russell's Snapper (*Lutjanus russelli*).

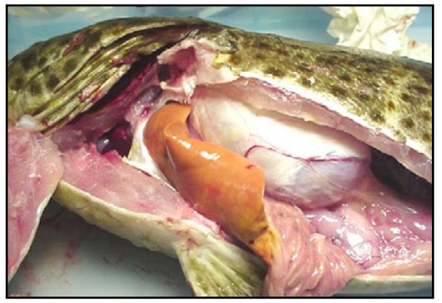


Figure 487. Green grouper (*Epinephelus coioides*) with yellow fatty liver.

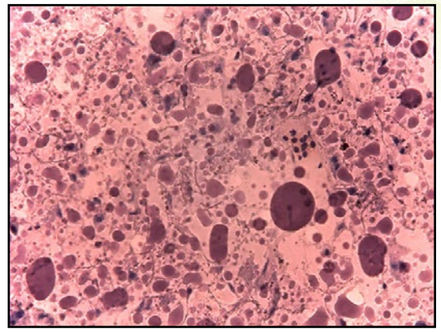


Figure 488. Green grouper (*Epinephelus coioides*) - Sudan Black liver showing fat stained as black.

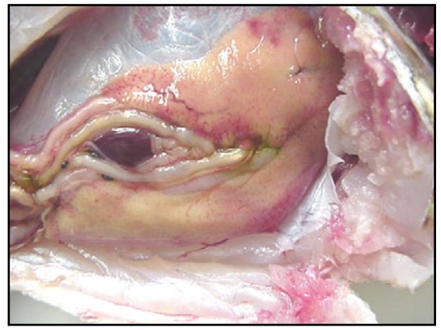


Figure 489. Tiger grouper (*Epinephelus fuscoguttatus*) with very pale fatty liver; endosulphan toxicosis involvement.

Epidemiology

The dietary fat percentage requirement for fish varies from 15-20% in trout fingerlings to less than 12% in tilapia (Lovell, 1989). Warm water marine fish generally grow well with a dietary lipid level of 8-12%, provided that essential fatty acid (EFA) requirements of DHA/EPA are satisfied (Chen *et al.*, 2002).

Pathophysiology

Fatty liver syndrome:

- Can be normal in species that naturally accumulate lipid in the liver.
- Can result in fatty degeneration due to the depletion of antioxidant Vitamin E and other vitamins, e.g., Vitamin C, which are normally added to commercial feeds. Prolonged storage, and high temperatures and humidity result in loss of antioxidants and this results in increased production of free radical compounds associated with rancidity of dietary fats. Vitamin E deficiency causes increased

breakdown of cell membranes including those of erythrocytes leading to anaemia (Stoskopf, 1993; Roberts, 2001). Myopathy may occur with Vitamin E deficiency.

- Can be due to essential fatty acids deficiency, which causes extensive hepatic lipid infiltration (Roberts, 2001). Liver failure results in a reduction in the secretion of haemopoietin (Halver and Hardy, 2002) and subsequent anaemia.
- Has also been reported in toxicoses, e.g., due to exposure to pesticides. However it is a non-specific change (Ribelin *et al.*, 1975).

Diagnosis

Grossly the liver may be enlarged and is greasy on the cut surface as well as being very pale. Either Sudan Black (Fig. 488) or Oil-Red-O (Fig. 492) staining of fresh frozen liver sections will reveal the degree of fatty infiltration. Histologically, signs of liver degeneration are:

- Extensive fatty vacuolation of hepatocytes (Fig. 491).
- Nuclear atrophy – nuclei appear small and pin-point in the centre of an enlarged lipid-filled hepatocyte.
- Eosinophilic droplets in the hepatocyte (Fig. 490).
- Hepatocyte cell membrane disruption (Fig. 490).
- Liver ceroid accumulation.

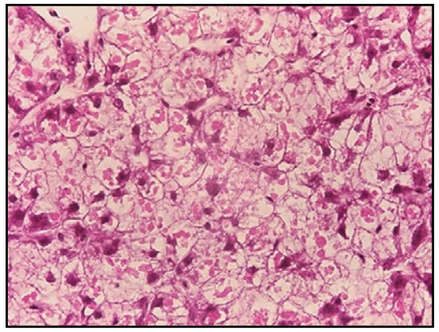


Figure 490. Grouper (*Epinephelus* sp.) with fatty liver degeneration; note the eosinophilic droplets and loss of clear hepatocyte cell membranes.

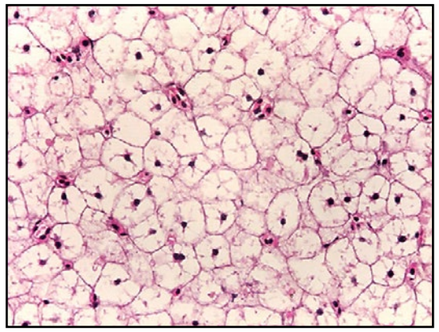


Figure 491. Tiger grouper (*Epinephelus fuscoguttatus*) liver with pinpoint nuclei and degeneration from endosulphan toxicosis.

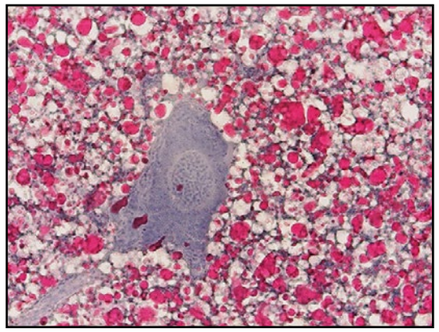


Figure 492. Red-drum (*Sciaenops ocellatus*) with heavy lipid infiltration of liver; Oil-Red-O stain.

Control and Prevention

Management of fatty liver syndrome involves:

- Provide appropriate levels of dietary lipids for the species of fish
- Provide essential fatty acid requirements
- Prevent the rancidity of feeds through proper storage conditions by avoiding high temperatures or humidity and prolonged storage before use; high levels of antioxidants (Vitamin E or ethyoxyquin) to prevent rancidity (NRC, 1993)
- Supplement the diet with choline chloride to promote fat clearance in the liver (Stoskopf, 1993)

Physical Deformities

Farm History

Physical deformities in fish occur infrequently but may be associated with particular batches of fish. Deformities can be related to:

- Physical trauma or physiological stress
- Genetic abnormalities
- Nutritional factors
- Pollutant exposure
- Infectious disease

It can be difficult to reach a definitive diagnosis for fish deformities but the approach needs to consider ruling out each possible group of causative factors. The following discussion provides a general framework in which to begin the process of elimination for cases presented (Figs. 493-497).

Physical Trauma or Physiological Stress

Impact: These occur occasionally depending on the species and aquaculture system, husbandry or hatchery methods employed. It increases morbidity with loss of marketable stocks.

Causes: They are associated with predator and competitor attacks, mechanical shocks (ova) and electrocution. There may be fracture and/or dislocation of spinal vertebrae producing a bent fish. Incorrect egg hatchery incubation conditions (incorrect temperature, dissolved oxygen, light, etc.) and other water quality factors lead to fish embryo and fry developmental abnormalities.

Epidemiology: Some species are naturally aggressive towards smaller cohort members, e.g., barramundi and Murray cod. Electrofishing may catch fish with spinal fractures due to the excessive current employed.

Symptoms: Attack trauma produces damaged fins and body injury with jaw/teeth marks. Ova shocks produce twins, pugheads and spinal curvatures. Electric shocks produce spinal fractures and curvatures. Blackening of the body skin distal to the spinal trauma site is due to blockage of autonomic innervation.

Management: Identify and correct the cause(s), e.g., regular grading, predator protection (seal nets around sea pens), improve husbandry and handling techniques or electrical wiring safety, optimise water quality conditions in hatcheries

Genetic Abnormalities

Impact: These are associated with using a small, restricted or non-renewed genetic pool leading to increased risk of inbreeding.

Causes: Genetic linked abnormalities are expressed in new generations of fish.

Epidemiology: Genetic abnormalities may be passed onto successive generations resulting in increasing prevalence rates of the deformities.

Symptoms: Twins, pugheads, cross bite, spinal curvatures, double fins and opercular deformities

Management: Increase genetic diversity in the broodstocks by bringing in and crossing with new varieties of the species. Genetic tests can be done to assess genetic diversity. Avoid breeding from brood lines that show an increased incidence of deformed young fish.

Nutritional Factors

Impact: Increased morbidity and production losses from reduced marketability of deformed fish although mortality may not be significant

Causes: Deficiencies in Vitamins C, E and A, magnesium, tryptophan and phosphorus, and Vitamin A toxicity

Epidemiology: Deformities are associated with the use of problematic feed batches over a period of time especially in young growing fish. Inappropriate storage of feeds, inadequately formulated feedstuffs or use of expired feed batches may be involved.

Symptoms: Vitamin E deficiency causes spinal deformities due to myopathy. Vitamin C and magnesium deficiencies cause spinal deformities due to vertebral weakness. Vitamin A deficiency causes opercular curling; toxicity causes spinal exostoses and deformities. Tryptophan deficiency causes spinal curvatures. Spinal deformities include scoliosis (lateral curvature) and lordosis (longitudinal curvature). Phosphorus deficiency produces distorted bone growth, particularly seen as poor gill formation and jaw deformity where fish appear as ‘screamers’ because the jaw cannot close properly. Spinal deformities have been

reported in Atlantic salmon because of the reduced availability of phosphorus that has been bound to plant phytates in commercial diets (Sullivan *et al.*, 2007).

Management: Identify and correct the nutritional imbalance, improve the feed storage conditions.

Pollutant Exposure

Impact: Deformities of wild-caught fish is not uncommon and are reported to be associated with polluted sites although a direct causal link can often be difficult to prove.

Causes: Toxic compounds include all man-made and naturally occurring substances such as lead, cadmium, zinc, organochlorines, organophosphate pesticides, polychlorinated biphenyls (PCBs), and pollutant mixtures in water ways.

Epidemiology: Exposures to toxins with acute deformities are possibly due to spinal fractures from muscular spasms. Exposures to toxins of young fish in developmental stages can lead to skeletal growth anomalies.

Symptoms: Lead causes black tails and spinal curvature. Cadmium and zinc can cause muscle spasm-related spinal fracture. Organochlorines (toxaphene, chlordecone) can cause muscle spasm-related spinal fracture or weakened vertebral fractures. PCBs can cause weakened vertebral fractures. Organophosphates (trichlorphon, malathion) can cause muscle spasm-related spinal fracture. General pollution can be associated with fin ray abnormalities, tumours and ulcers.

Management: Most spinal abnormalities are not amenable to treatment. Management is focused on identifying the source of toxin(s) through a series of analytic tests on samples of fish, water, feed or sediment, particularly as part of a fish kill investigation. Exposure experiments with control and treated fish are often required to define cause and effect relationships. Elimination of infectious or parasitic causes of such deformities will form part of the goals of an investigation.

Infectious Disease

Impact: Deformed fish have poor growth, survival and marketability. The agent is infectious and the problem can persist on a farm.

Causes: Viruses: Infectious Haematopoietic Necrosis Virus (IHNV), Ramirez dwarf cichlid virus and Epizootic Haematopoietic Necrosis Virus (EHNV)

Bacteria: *Streptococcus iniae* and *Mycobacteria* sp.

Protozoa: *Myxobolus cerebralis, M. ellipsoides, M. spinacurvatura, Triangula percae, Hoferellus cyprini, Kudoa* sp. and microsporidians

Fungi: *Ichthyophonus hoferi*

Epidemiology: Deformities are most likely when infection occurs in the fry or young stages of the fish life cycle. Deformities in older fish can be due to tissue inflammation of the musculoskeletal tissues and other organs as part of more chronic pathology following the survival of the acute phase of the infection.

Symptoms: Spinal curvature is the main pathology. Jaw, cranial and gill deformities are associated with *Myxobolus cerebralis.* Spinal deformities in mycobacteriosis are due to granulomas in the spinal tissues. Transient scoliosis occurs with Ramirez dwarf cichlid virus infections.

Management: This depends on the pathogen but will include exclusion of infected stocks from the farm and disinfection. For *Myxobolus cerebralis* (whirling disease), the use of concrete raceways to avoid exposure of the fish to the intermediate host (tubificid worm) will disrupt the life cycle of *M. cerebralis.* The process of ruling out other non-infectious causes of the deformities must also be carried out.

Figure 493. Deformed heads of green grouper *(Epinephelus coioides)* fingerlings *(Epinephelus coioides)* reported in 60% of fish upon arrival for stocking at farm; probable genetic or nutritional cause.

Figure 494. Deformation and variation in head and body shape of green grouper *(Epinephelus coioides)* fingerlings; probable genetic or nutritional cause.

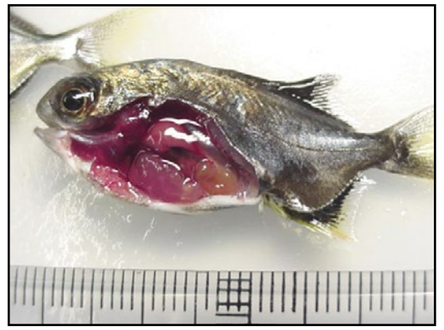


Figure 495. Pompano (*Trachinotus blochii*) fingerling with ventral abdominal deformation in 33% of the sample; damage of the fry while in the embryo developmental stage by adverse environmental factors (high temperatures, toxic compounds), nutritional deficiencies (e.g., Vitamin C) or genetic inbreeding.

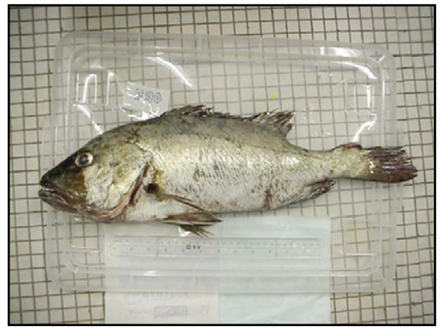


Figure 496. Mangrove snapper (*Lutjanus argimenticulatus*) with tail peduncle deformity probably from trauma or healed deep ulceration due to bacterial infection as this is an old fish.

Figure 497. Discus (*Symphysodon* spp.) juvenile with large missing part on the ventral caudal area; possible genetic cause.

Figure 498. Discus (*Symphysodon* spp.) juvenile with incomplete operculum; possible genetic cause.

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Index

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z

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