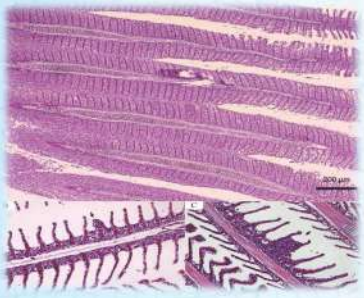
THE NEOTIA UNIVERSITY

ज्ञानम् आत्म प्रदीपाय





Practical Manual

Edited by

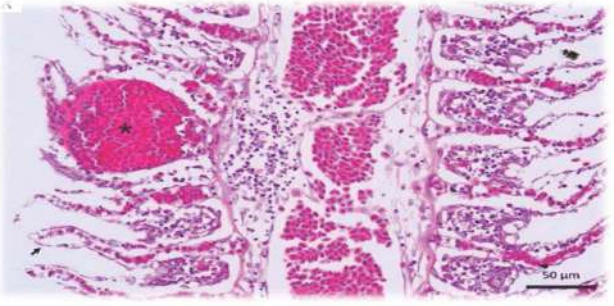
Dr. Sudeshna Sarker

Subject: Fish and Shellfish Pathology

Course No.: CC-FSP 432

Bachelor of Fishery Science (B. F. Sc.)

2nd Year 2nd Semester



Department of Fishery
School of Agriculture and Allied Sciences (SAAS)
The Neotia Uniersity
Jhinga, P.O.- Amira, Diamond Harbour Road, District- 24 Pgs (S), PIN- 743368, West Bengal

TABLE OF CONTENTS

CHAPTER 1

Sample Collection and Submission Proforma

Sample Submission Form

Date COLLECTED: _____

Facility/contact person: _____

Lot (BROOD YEAR/STOCK/SPECIES): _____

Life stage: _____

Sex if applicable: _____

Date outbreak noticed: _____

Problem history: _____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

Recent medications: _____

Video Link: https://youtu.be/Rttu1ZBRyEs

CHAPTER 2
Live and Post Mortem Examination of Finfish

Principle

An abnormality in a fish could be recognised by observation of various clinical signs such as behavioural changes, morphological abnormalities, presence of external parasites, internal examination followed by histopathological, bacteriological, virological and mycological analysis of fish tissues. Diagnosis of the infection could be done from various observations and tests results in combination. While in some cases the infection can be easily diagnosed e.g. by the presence of parasites, in other cases, battery of tests may be required to find the aetiology of infection. The present experiment is level 1 diagnosis with observations followed by sampling of various tissues.

Materials

- Fish
- Dissection kit
- Microscope
- Glass slides
- Sterile TSA plates
- Sterile containers
- Anaesthetic (MS222 or benzocaine)
- Syringe with 24 g x 1" needle
- Fixative (NBF/Bouin's Fluid)

Procedure

Observation of behavioural changes

Wherever possible fish sampled for disease diagnosis should be examined for any behavioural abnormalities. This may include listlessness, swimming near surface sometimes putting the head out of water, gulping of air, loss of balance, corkscrew swimming, belly up rolling motion, flashing and scraping, lethargic movement, increased respiration, increased feed uptake followed by anorexia and reduced reflexes. Make a note of the above abnormalities, if any.

Gross examination

Examination of fish can be done on sacrificed specimens. Kill the fish just prior to examination by decapitating - severance of spinal cord behind the head or anaesthetic over dose. Tricaine methanesulphonate (MS – 222) 1: 1000 or Benzocaine 0.1g/4l. (dissolve benzocaine in small quantity of acetone and then dissolve in water) will anaesthetise the fish within 5-10 minutes

Examine the external surface of the fish for any lesions or abnormalities. This may include damaged skin, ulcers, reddening and haemorrhages, changed colouration, abdominal swelling, abnormal eyes with exophthalmia, cloudiness and gas bubbles, skeletal deformities, eroded fins, abnormal growths and increased mucus production. The skin surfaces should also be examined for the presence of parasites. Gills are to be examined for its colour, lesions, presence of parasites and excessive mucus. Healthy gills should be red with a symmetrically outer periphery.

Video Link: *https://youtu.be/SItEuymGkqY*
https://youtu.be/-cEFAtqDeS0

CHAPTER 3
Sample Collection of Finfish

Order of sample collection

1. Blood sample
2. Fresh preparations from gill and skin.
3. Samples for mycology
4. Samples for bacteriology from the kidney
5. Gram stain of kidney smear
6. Samples for histopathology
7. Samples for virology
8. Fish should be kept moist until it is examined for the presence of parasites.

Blood collection

Blood should be collected as soon as possible once the fish is received. Place the fish on its side and insert the needle behind the anal fin at an angle forward until it touches the spinal cord. Constantly apply a negative pressure and reposition or rotate the needle to withdraw blood. Once blood start flowing keep the negative pressure constant and do not shake or change the position of the needle. Blood will continuously flow into the syringe. Prepare blood smear for further analysis. Make a thin film of blood (dealt separately in chapter 3).

Fresh Preparations

It is essential to keep the fish moist preferably with water from which it was removed. Many of the parasites are detected primarily by their movement. If they are allowed to dry out or placed in chlorinated tap water they will die rapidly.

Fresh skin scrapings are obtained by gently scrapping the surface of the fish especially behind fins e.g. dorsal and pectoral with a scalpel blade. The collected scrapings consisting of mucus with epithelial cells is transferred to a glass slide with a drop of the water in which the fish was kept and cover-slipped. The slide is then examined under the microscope for the presence of parasites.

A portion of the gill primary lamella is then cut and removed from the gills and placed on a glass slide and cover-slipped with a drop of water. The tissue is then moved and pressed slightly for observing the secondary lamellae. The preparation is then examined under the microscope.

Internal Observations

The internal organs of the fish are examined after cutting open the body along the ventral surface. Healthy fish should show normal visceral organs without any free fluid

accumulation or peritoneal damages (peritonitis). The internal organs should be examined for the presence of parasites, internal hemorrhages, petechiale, hyperemia and any other abnormal symptoms including swelling of the organs, discoloration and abnormal growths .

Sample of Mycology

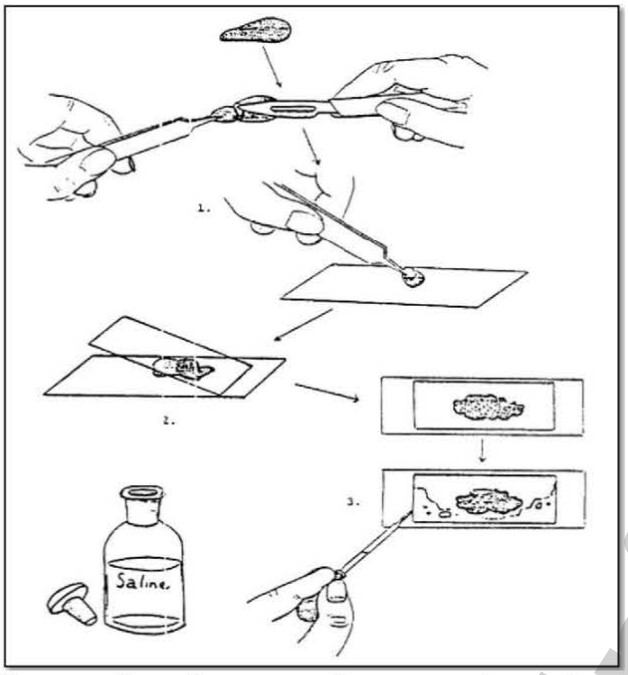
The samples for mycology is collected from surface lesions which show fungal infection and hyphae. After surface disinfection the tissue with fungal hyphae are collected and taken for culturing the fungi. For analysis of deep penetrating fungi, the muscle tissue below the lesion is taken for analysis (Culture of fungi is dealt separately)

Sample of Bacteriology

Once external examination or sampling has been carried out, the body surface is cut open to expose the internal organs. In the event of any external lesions,bacteriological samples could be collected from the lesions after surface disinfection before opening the body cavity. Care must be taken not to puncture any part of the intestinal tract. In the absence of any visible internal lesions, a sample of kidney is taken and inoculated onto suitable agar medium.If no preliminary diagnosis could be made of the infection, use TSA (trypticase soya agar) for isolation of the bacteria. Material from other organs e.g. liver,spleen, heart can be taken if any abnormality is evident. The surface of any internal organs to be sampled should be seared with a hot scalpel blade before insertion of a sterile loop but in the case of very small fish, this may not always be possible. Agar plates containing the streaked out samples should be incubated and examined daily for any evidence of growth. The majority of fish pathogens will grow on TSA within 7 days.

Petri dishes containing solid medium (agar) are used to provide a large surface area for the cultivation of microorganisms. Inoculation of an agar plate is carried out using streak plate technique. This involves diluting the culture or other sample, e.g. kidney material, by smearing it across the surface of the agar. Organisms present in the samples will be separated and after suitable incubation, each organism present will give rise to a colony. Although this colony contains many millions of organisms they will have all originated from one, and therefore all organisms in one colony should be identical.

By using this method, organisms can be cultured in the laboratory. However, if as mixed culture is present, it will become apparent on plating out. This is essential before starting identification procedures, as methods are only valid when carried out on pure cultures i.e. cultures containing only one type of organism. Care should be taken to ensure that plates used for this purpose are dry. Avoid unnecessary exposure of the agar surface to potential contamination from the environment throughout the procedure. Biochemical characterisation of bacteria will be carried out according to Bergey's Manual (Bergey, 2001).



Preparation of compression smear (squash preparation) from internal organs

Kidney smears

Direct examination of Gram stained kidney smears may give an indication of bacterial septicaemia. A small portion of kidney is emulsified in a loopful of sterile physiological saline on a microscope slide, air dried then fixed by passing the slide through a Bunsen flame several times. Direct observation of slide is carried out using the 40x and 100x (oil immersion) objectives after Gram's staining.

Samples for histology

Samples for histology must be taken as soon as possible after death of the fish and fixed immediately, preferably in neutral buffered formalin or Bouin's solution (Picric acid+formaldehyde+acetic acid).

The organs sampled depend on the nature of the problem however, here all the major organs can be sampled e.g. gill, skin including muscle around lateral line, heart, liver, pancreas/ gut, spleen and kidney. The samples should be sufficiently small to allow the fixative to penetrate rapidly through the tissue. Therefore the dimensions of the tissue removed should be less than 7.5 mm. The tissues should be placed in at least 10 times their volume of fixative i.e. one part tissue to ten parts fixative. The tissue can be kept in formalin for prolonged periods but they should be stored in such a way as to avoid any portion of the tissues remaining out of the fixative. (Histological analysis is dealt separately in chapter 6)

Samples for virology

From larger fish 1 g of each tissue is collected for processing. Once dissected out the tissue should be maintained in ice without direct contact. In case of smaller fish, the tissues from each organ could be pooled to obtain 1 g in total. The tissue inoculation to the cell culture flasks after processing should be done within 24 hours in ice.

Tissues from a maximum of 10 fry or fingerlings may be pooled and treated as one sample. When larger fish are sampled, tissues from a maximum of 5 fish may be pooled and treated as one sample.

Transportation / storage of samples

Pooled samples must be kept in a clearly labelled leak proof container. These should be packaged in separate sealed plastic bags for each sample. Samples must be transported only on ice without freezing to a laboratory as quickly as possible. The samples should be packaged in a robust container and laboratory should be notified about despatch. Samples must reach the laboratory and should be processed within 48 hours of collection (Processing of tissues for virology is dealt separately)

Record keeping

All observations should be noted down with specification and pictorial records need to be stored separately.

Samples for culture independent PCR analysis

Samples for PCR analysis can be transported on ice and stored in -20 degree C or should be fixed in either 95% or 70% ethanol. It is best to use cold fixative that has been stored in the freezer or kept on ice, as this helps arrest autolysis and secondary microbial proliferation, as the tissues are preserved.

Video link: https://youtu.be/Sf8AxvkAhYo
https://youtu.be/TlGeYLxNTUc

CHAPTER 4

Blood Sampling

Introduction

This template is intended for use by instructors to train the students in the blood sampling of finfish. Templates are used to provide the minimum requirements necessary in a trainingexercise, but the instructor may add additional material as deemed necessary.

An experienced instructor must demonstrate the methods outlined in this template and trainees must be deemed qualified in carrying out one or more of the procedures before they are permitted to blood sample without an instructor present. Hands-on training of staff is a requirement for facility approval by the Canadian Council on Animal Care (CCAC), of which DFO is a member. This template is part of a comprehensive DFO Science Branch series on training for users of aquatic research animals.

Rationale

Scientific study often requires that fish be blood sampled for reasons such as haematology, clinical chemistry parameters, bacteriology, parasitological investigation, packed cell volume, etc. Investigators have an ethical obligation to minimize the pain and/or distress of all laboratory animals undergoing laboratory-sampling techniques. Only those methods deemed acceptable by the CCAC should be employed.

Authority

The staff/consultant veterinarian or Animal Care Committee is responsible for providing information about blood sampling methods of the fish species used for scientific study in their respective regions. The Animal Care Committee may delegate training of these procedures to an instructor who has demonstrated knowledge and experience in the areas of anatomy and anaesthesia and has demonstrated excellent survival of fish that have been blood sampled using the techniques outlined in this training template. Staff must be trained in the proper blood sampling techniques for the fish species and size being studied prior to initiation of any blood sampling procedure(s).

Goal of this training exercise

Learn the methods to humanely remove blood samples from anaesthetizedf

Understand the consequences of each method of blood sampling on the fish and thus the potential impact of the method selected and the sample collected.

1. Understand the function/physiology of the circulatory system within a fish.
2. Demonstrate gentle handling techniques of fish when live fish are used in thissection.
3. Understand proper disinfection techniques to use after completing theprocedure (provide biosecurity SOPs).

Details of the Procedure

Introductory

Methods of blood sampling, that may be included in the training session, are:

1. Blood sampling by tail ablation of small fish that have been euthanized.
2. Blood sampling by caudal venous puncture.
3. Blood sampling by dorsal aorta puncture.

Advanced:

4. Blood sampling by heart puncture.

Time estimate for completing the hands on exercise Set up: 1 hour
Instruction and Training: 2 hours

Equipment Required

1. Fish: number will depend on the number of trainees in a session;species used will depend on availability and requirements of thefacility.
2. TMS™ (plus $NaHCO_3$ buffer if anaesthetizing in freshwater)
3. Dip net
4. Container for anaesthetic bath
5. Air stones and compressed air or oxygen
6. Thermometer
7. Gloves and splash glasses to be worn when handling fish oranaesthesia water
8. Disinfectant for clean up after blood sampling
9. Soft sponge or mat to prevent mucous sloughing during bloodsampling
10. Scalpel or sharp knife for tail ablation
11. Paper towel for containment of blood from ablation
12. Sterile needles or vacutainer needles (size will be dependent on thesize of the fish)
13. Syringe or vacutainer tube for collecting blood samples (size willdepend on the amount of blood to collect and the size of the fish)
14. Vacutainer holder
15. Tube racks for blood collection tubes
16. Hematocrit tubes
17. Critoseal
18. Sharps container

Procedure

The instructor should demonstrate all the procedures prior to allowing the trainees to attempt them. Fish sacrificed in the euthanasia template make excellent teaching specimens for the trainees to practice their techniques.

Instructors will need to use their own best judgement in choosing which procedures are appropriate with the common species used at their researchfacility. It is recommended that all procedures be taught to trainees so that they have multiple options at their disposal when faced with the need to sample the blood of fish of different species.

Introductory
Equipment for Blood Collection

Human Safety Measures

> Discuss human safety measures while discussing the equipment usedfor blood sampling.

> Wear gloves at all times when handling blood collection equipment.While this will not prevent accidents, it may reduce the risk of contamination.

> Never attempt to re-sheath needles. Always discard them into a sharpscontainer immediately after use.

> Never overfill the sharps container. Adhere to the recommended fillline on the sharps container.
> Place the sharps container in the area you are working to avoidtransporting used needles outside of your work area.

Ensure the area you are working is clean and all the equipment need tocarry out the procedure is readily available. Allow enough workspace to safely obtain the blood sample without contaminating the blood sampling supplies should the fish exhibit common reflex movements upon needle insertion. For blood collection in the field it is suggested to spread a fine mesh net under the entire work area to prevent loss of supplies (particularly sharps) that may accidentally drop while sampling.

Equipment Options

- Present equipment options available for blood collection (i.e. needlesof various gauges and length, multiple syringe types and sizes, haematocrit tubes and critoseal, vacutainer needles and tubes, etc.).
- Emphasize the relationship between needle gauge and needle size (e.g. a 20 gauge needle is larger than a 30 gauge needle).
- Discuss safety equipment and how it is to be used (i.e. tube racks arefor holding tubes after filling with blood, sharps containers are for discarding used needles, etc.).
- Identify the proper needle gauge and length required for bloodsampling the fish used in this training session.
- Discuss the common types and sizes of vacutainer tubes and chose thetype and size appropriate for the fish used in this training session. Trainees should consult with the principal investigator to determine the appropriate tubes to use when blood samples are collected for research purposes. Examples of vacutainer tubes include, but are not limited to the following:
- Red top: contains no additives. Used for serum collection, bacteriology, and parasitological examination.
- Green top: contains the anticoagulant lithium or sodium heparin. Intended for clinical chemistry parameters and plasma collection.
- Purple top: contains the anticoagulant EDTA. Intended for whole blood collection for haematology. EDTA will interfere with some tests and is therefore not a suitable sample tube for blood chemical analysis.
- Grey top: contains the additive sodium fluoride. Intended forglucose measurements.
- Light blue top: contains the additive sodium citrate. Intended forcoagulation studies.
- Select the appropriate tube or syringe size for the fish used in the training session. Too large a tube or syringe can significantly reduce the blood reserve in smaller fish causing excess stress and/or death. Paediatric tubes or 1 ml syringes should be used when blood sampling smaller fish.
- Instruct trainees in the proper method to attach and detach needles to syringes or vacutainer tubes.
- Stress the importance of feeling comfortable with the bleedingapparatus prior to insertion into a living fish.

- Trainees should practice moving the plunger up and down in the syringe. A good practice tool is having the trainees insert a syringe with attached needle into an orange and extract a small amount of juice.
- Trainees should practice inserting a vacutainer into the vacutainer holder (with attached needle) without breaking the vacuum seal. A break in the vacuum seal in air makes the tube useless for blood collection, as the blood will not aspirate once in the vein or aorta of thefish.

Note: The following procedures and photographs have been adapted with permission of the author from Chapter 4. Aseptic Bacterial Examination of Finfish in: Finfish and Shellfish Bacteriology Manual-Techniques and Procedures, 2004. Whitman K.A. (author); Blackwell Publishing, Iowa StatePress, Iowa, USA.

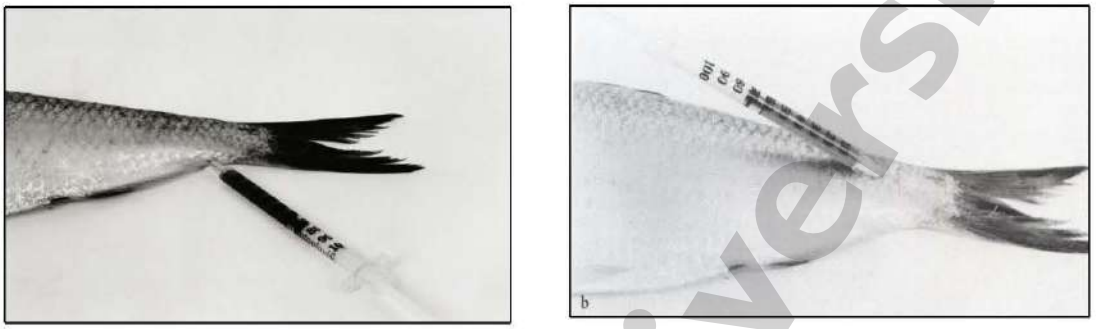
Tail Ablation

- Fish that are too small to bleed with a syringe and needle or a vacutainer system may necessitate lethal sampling by tail ablation.
- Euthanize a fish. Refer to the Euthanasia Template for acceptedmethods of euthanasia.

Blood collection by Tail Ablation

- Sever the caudal peduncle with a scalpel blade or sharp knife.
- Fill a hematocrit tube with the blood as it flows from the caudalvein.
- Plug one end of the haematocrit tube with critoseal.
- Dispose of the fish carcass and waste as outlined in facility SOP.

Caudal Venous Puncture



Blood collection using the caudal vein

- Insert a needle attached to a syringe or a vacutainer system under the skin of the ventral midline of the caudal peduncle ofan anaesthetized or freshly euthanized fish.
- Alternatively, a lateral approach can be used by inserting the needle under the scales of the mid portion of the tail just below.
- The lateral line at a 45° angle to the long axis of the fish in acranial direction.
- Ease the needle toward the vertebral column until you reach thebase of the column.
- Withdraw the needle a fraction of a millimetre, and obtain theblood sample.
- Remove and discard the needle in a sharps container.
- Return the fish to a recovery bath or its home tank and monitorthe recovery process.
- Prepare the blood sample for processing as instructed by theinstructor.
- Clean the work area as outlined in the facility SOP.

Dorsal Aorta Puncture

Blood collection using the dorsal aorta

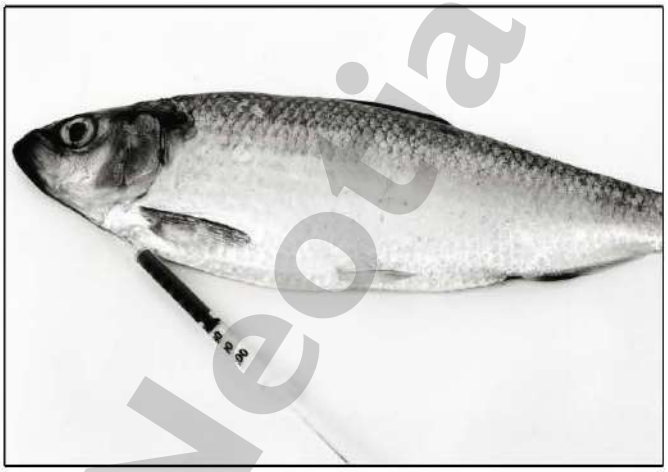
- Insert the needle attached to a syringe or a vacutainer system, bevel pointing upward, along the dorsal midline of the mouth, just past the juncture of the second gill arch of an anaesthetized or freshly euthanized fish.
- Collect a blood sample.

- Remove and discard the needle in a sharps container.
- Return the fish to a recovery bath or its home tank and monitorthe recovery process.
- Prepare the blood sample for processing as instructed by theinstructor.
- Clean the work area as outlined in the facility SOP.

Advanced

- Blood collection using the heart puncture method is often performed on euthanized fish, though it can be performed successfully on anaesthetized fish with proper training.
- Instructors should demonstrate this procedure on several fish to ensure the trainee is cognitive of the potential risks associated with this method.
- Trainees should not be permitted to perform this procedure on live fish without a firm grasp of the anatomy and physiology of the heart.
- It is helpful for trainees to dissect a sacrificed specimen to observe the location (bulbous arteriosus) where the needle is to be inserted and determine the potential trauma a fish's heart can undergo after blood sampling.
- Trainees should practice on a number of euthanized specimens to practice their technique and gain confidence prior to attempting a live specimen.

Heart Puncture



Blood collection using the heart puncture technique

- Hold the needle attached to a syringe or vacutainer system perpendicular to the skin and insert the needle slightly below the tip of the V-shaped notch formed by the gill cover and theisthmus of an anaesthetized or freshly euthanized fish.
- Collect the blood as the needle enters the bulbous arteriosus.
- Remove and discard the needle in a sharps container.
- Return the fish to a recovery bath or its home tank and monitorthe recovery process.
- Prepare the blood sample for processing as instructed by theinstructor.
- Clean the blood sample area as outlined in the facility SOP.

After the training session

- Increase monitoring for 2 – 3 weeks after handling if live fish were used for the training.

- Trainees should have clear instructions for needle/sharps disposal and carcass disposal.
- Anaesthesia baths must be disposed of in accordance with local waste management regulations.
- Disinfect the area where fish were handled (provide trainees with site bio-security SOP).
- Trainees must wash hands with disinfectant soap.
- Update inventory records to reflect the number of fish euthanized for this session (if any).
- Update drug use records to include anaesthetic use.

Blood Collection Guidelines

- No more than 0.1% of the fish's body weight (i.e. 1ml/kg) should be removed from a fish that will be recovered from blood collection.
- Fish greater than 200 grams can be recovered successfully following blood collection (dependant on the health status of the fish prior to blood sampling).
- Fish smaller than 200 grams may have to be sacrificed to ensure an adequate blood volume is collected.
- Fish must be allowed adequate time to recover and regenerate blood volume if serial blood samples are to be collected.
- Blood collected for haematology and clinical chemistry parameters must be taken from living fish. Care must be taken to avoid contamination of the sample with tissue fluid.
- Fish blood clots very rapidly. The type of anticoagulant used should be determined in consultation with the principal investigator. For some parameters an anticoagulant will not be required. Examples of anticoagulants include:

  EDTA (dipotassium ethylene diamine tetraacetic acid): acidifies blood, binds divalent cations lowering calcium measurements in the sample.

  Disodium oxlalate: raises blood pH and sodium levels.

  Trisodium citrate: raises blood pH and sodium levels.

  Lithium or Sodium heparin: can cause erythrocyte clumping but does not interfere with divalent cation measurements and has less effect on blood pH. Lithium heparin does not interfere with sodium levels.

- Equipment used for blood collection should be scaled to the size of the fish and the expected blood volume to be collected.
- The length and gauge of a needle used for blood collection will depend on the size and species of the fish being sampled. (E.g. a 22 gauge, 1 ½ inch needle is most commonly used for salmonids averaging 1 kg).
- Only sterile needles should be used for blood collection.

- The choice of using a vacutainer system or a syringe and needle is based purely on preference.

Blood Sample Handling:

- Blood samples should be processed within 30-60 minutes after collection. The time between blood sampling and laboratory evaluation of the sample collected is critical. Cells can swell and rupture, and some parameters may not be stable during the time between collection and processing.
- The handling and storage of samples after processing will dependon their purpose. Check with the principal investigator to determine how to process the samples.

Blood collection: *https://youtu.be/scfkQhHTYqg*
https://youtu.be/9kpxB5bnC7I

CHAPTER 5
Blood Smear Preparation

Preparation of blood smears for observing different cellular component of fish blood

Materials required

- Blood sample collected from fish
- Needle
- Glass Slide
- Methanol
- Giemsa Stain
- Oil Immersion Microscope

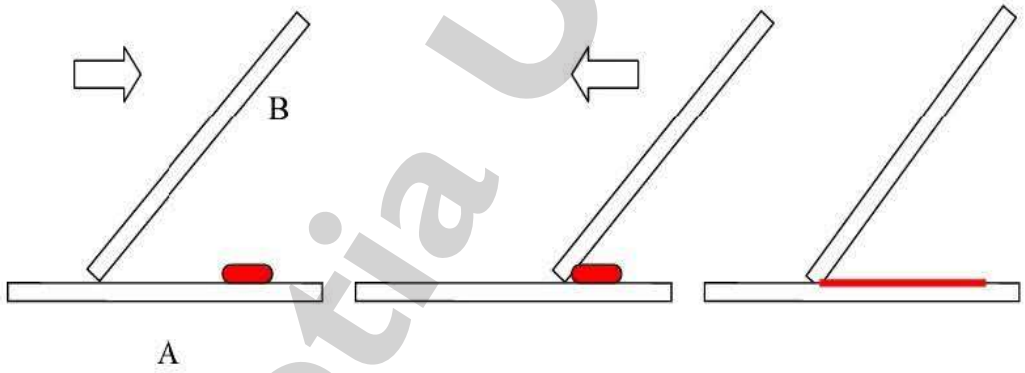
Procedure

The preparation of blood films on slides

1. Place a small drop of blood in the centre of a slide about 1-2 cm from one end.
2. Keep the spreading slide at an angle of above 45 ° to the slide and move backward to make contact with the blood drop.
3. Spread out the blood drop should quickly along the line of contact by using the spreader slide.

How to prepare blood smear

1. On slide "A" express a drop of blood about one-half inch from the end.
2. The edge of a second slide "B" is placed on the surface of slide "A" at about a 45° angle and is moved backward (to the right in the diagram) until contact with the drop of blood.
3. Contact with the blood will cause the drop to spread along the edge of slide "B" due to capillary action. Slide "B" is then pushed forward (left in the diagram), being careful to keep the edge pressed uniformly against the surface of slide "A".
4. The size of the drop of blood and acuteness of the angle formed between the slides will determine the thickness of the film. A more acute angle results in a thicker film.
5. The smear is allowed to air dry for transport in a slide box and later staining.



Staining

1. Air-dry the blood smear (If stain is added to wet smear, the colour of stained smear will be greenish and it will hinder the acidic and basic colour of different cells).
2. Fix the smear for 3-5 minutes in methanol
3. Cover the smear with Giems stain.
4. Leave the stain for 30-40 minutes.
5. Wash the slide gently with distilled water to remove the extra stain and stain particles and then under slow running tap water.
6. Air-dry the slide and observe it under oil immersion objective. The stained smear will be violet in colour.

Identification of different types of fish blood cells

- The cells should be observed under microscope (40X, 100X magnification) in oil immersion.
- Focus on the ideal zone of the smear.
- Move the slide to reach the other edge of the smear. Note down the observation.

Erythrocytes (RBCs)

Mature fish erythrocytes are oval in shape and are nucleated with abundant pale eosinophilic cytoplasm. They occur both as mature and immature forms. The size of the erythrocytes varies from 10 -11 m to 12-13 m . Immature erythrocytes are called reticulocytes and are larger than mature erythrocytes. The size of the nucleus is larger in immature ones.

Granulocytes

Neutrophils (polymorphonuclear): These are large cells with 9-13 m in size. Granules are fine and large in number with violet colour. Nucleus has many (2-5) lobes. Cytoplasm of these cells stains violet in Giemsa stain.

Eosinophils

Cell size 4.5–10 m. Granules coarse, less in number compared to neutrophils. Granules stains dark pink or red colour. Nucleus is usually bilobed and sometimes 3 lobed and spectacle shaped and is mostly placed at the periphery.

Basophils

Cell size 10-12 m. Granules are big and few in number. They overlap the nucleus and take up blue or purple colour. Big granules conceal nucleus. It is lighter in colour and is single or bi-lobed.

Agranulocytes

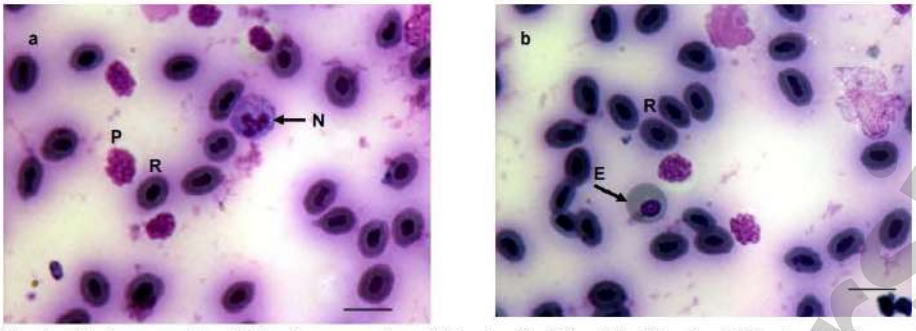
Small lymphocytes : Nucleus found or sometimes indented and it is large when compared to cell size. It occupies whole interior of the cell. Under the microscope, they look like purplish dots. Cytoplasm is very little and is placed at the periphery, light blue in colour. The size of these cells vary from 6 to 11 m .
Large lymphocytes : Size is larger than erythrocytes. Nucleus is either oval, round or kidney shaped, and placed in the centre. Chromatin material in nucleus is in clumps. There is abundant cytoplasm and it is light blue in colour.

Monocytes

Largest of all cells having a size range of 10 -14 m. Cytoplasm is abundant and peripherally placed while the nucleus is horse – shoe shaped and eccentrically placed. Chromatin material of nucleus is in network. The cytoplasm stain greyish blue in colour.

Thrombocytes

These are fusiform or oval in shape and have a thin cytoplasm around the nucleus. These are originating from spleen and are found in circulation unlike that of the mammals. The average size of the thrombocytes in catfish is 4 ´ 7 m to 5 ´ 13 m . These cells are primarily involved in the clotting process of blood.



Blood cells from a stained blood smear. a) red blood cells (R), white blood cell-Neutrophil (arrow-N) and Platelets (P); Bar= 10µm. b) red blood cells (R), Immature erythrocyte (arrow-E); Bar= 10µm.

Blood smear preparation and staining: https://youtu.be/KSs0SMfERuA
https://youtu.be/nbRUiWl2Qrs

CHAPTER 6

Live and Post Mortem Examination of Shellfish–I

(Sample a shrimp suspected of a disease for diagnosis of infection)

Principle

Clinical signs of infection in shrimp can be monitored by noting the behavioural changes and morphological abnormalities, presence of parasites, condition of the exoskeleton, followed by histopathological, bacteriological, mycological and molecular biological analysis of shrimp tissues.

Materials

- Shrimp
- Dissection kit
- Microscope
- Glass slides
- Sterile TSA plates
- Sterile containers
- Davidson fixative (1 L = 95% ethyl alcohol - 330 ml + Formalin (37% w/v formaldehyde in aqueous solution) - 220 ml + Glacial acetic acid - 115 ml + Distilled water - 335 ml). Store the fixative at room temperature (Avoid contact of the fixative with skin or eye)

Procedure

Clinical observation of shrimps

Clinical signs of infection in shrimp can be monitored by noting the behavioural changes and morphological abnormalities.

Major behavioural changes that can be observed in shrimp are:

- ✓ Reduced escape reflexes
- ✓ Swimming near pond edges or at the surfaces associated with lethargy
- ✓ Unusual aggregations
- ✓ Reduced preening activity and
- ✓ Increased feed consumption followed by anorexia.
- ✓ Beginning of mortalities is an indicator of infection. Depending on the pattern of mortalities and the observation of dead specimen, a preliminary diagnosis could be made. While uniform mortalities across the system point to adverse environmental conditions, irregular or sporadic mortality may be due to an infectious cause. The affected or dead shrimp should be removed from the system without any delay.

Collection of samples

Shrimp samples should be collected with minimum handling. For studying diseased shrimps, moribund, discoloured shrimp, displaying abnormal behaviour should be selected. Dead samples could be collected if dead recently and must be transported to the lab covered in polyethylene bags kept in ice in 24 hours. Random sampling is done for routine health status monitoring. Non-random sampling is done when disease is suspected.

All samples collected for disease diagnosis should be accompanied by the following supporting information wherever possible:

- ✧ Behavioural changes noticed for the last few days
- ✧ Brief history of the environmental parameters
- ✧ Clinical signs including external pathology and morphological symptoms
- ✧ Farming details followed like details of feed, feed consumption rates, previous disease history and any chemical treatments or other inputs used
- ✧ Mortality and morbidity pattern
- ✧ History of the farmed animals including origin, date of stocking and previous health status
- ✧ Samples collected for health surveillance should be large enough for screening and proper pathogen detection. Samples should be collected preferably by stratified random sampling to get a proper representation of the susceptible age and size groups of the animals. Surveillance sampling should be carried out in season best known to aggravate the incidence of the disease.

Examination of larvae and post larvae

Examine the specimen under dissecting microscope. Look for activity, gut contents, faecal strands, surface fouling, deformities, broken and missing appendages etc. for larvae and postlarvae. Select abnormal animals and transfer with a pipette to a glass slide and observe. Examine under low magnification objectives first, then with sub stage condenser set at reduced light for:

- ➢ Surface fouling of appendages and cuticle for filamentous bacteria and protozoans,particularly stalled
- ➢ Oral region for bacterial "plaques" (presumptive for Vibrio spp.).

- Larval mycosis - diagnosed by demonstration of hyphae, discharge tube, and motile zoospores.
- Muscle atrophy in abdomen, especially in 6th abdominal segment (muscle should fill half of space available).
- Hepatopancreas atrophy, vacuolisation, lipid droplets and BP inclusion bodies.
- "Bolitias blancas" (white balls) - sloughed hepatopancreas or midgut epithelial cells which gives a presumptive diagnosis of vibriosis.
- "Bolitias negras" (black balls) – poorly digested algae which indicates poor algae quality or possible vibriosis.
- Gregarine trophozoites in hepatopancreas or midgut cuticular deformities: bent or broken setae or spines.
- Melanized appendages tips or foci.
- Motile rod-shaped bacteria in hemocoel: vibriosis in most cases.

Direct examination of the shrimp tissue for BP, MBV or BMN:

Dissect out hepatopancreas using fine pointed dissecting needles and place hepatopancreas in a drop of clean seawater or saline on slide. Add small drop of 0.01% malachite green. Squash with coverslip, and examine over next 5 min for baculovirus occlusion bodies and / or hypertrophied hepatopancreas cell nuclei. BP and MBV occlusion bodies take up green stain rapidly compared to other cellular materials. MBV infection is characterised by single or multiple spherical occlusion bodies, which are intranuclear, and range in diameter from 0.1 to 20 µ. BP infection on the other hand is demonstrated by single tetrahedral occlusion bodies. BMN infection is identified by the presence of hypertrophied nuclei with single eosinophilic inclusion body that fills the nucleus.

Video Link: *https://youtu.be/NUGq9TvfycM*
https://youtu.be/md-qAr9yGIM

CHAPTER 7

Live and Post Mortem Examination of Shellfish–II

Examination procedures for juveniles and sub adults

External signs and symptoms

- Abnormal moulting also indicates a diseased condition. Lack of feed in the gut is an indicator of potential infection.
- Holding the shrimp against a light to show the gut in the tail segments can be used to check the gut contents.
- Healthy shrimp will be with characteristic pigmentation having pale bluish, bright and clear cuticle with proper hardness.
- Exoskeleton with brownish discoloration and occasional mat-like appearance denote protozoan fouling.
- In case of soft shell syndrome, the infected shrimp will have a soft exoskeleton and a hard rostral spine that will be different from the moulted shrimp, which would have a soft shell and a soft rostrum.
- Bacterial infections in shrimps are characterised by melanized brownish / black ended areas or blisters on the exoskeleton or appendages.
- Antennae cut, breakage of the cuticle, necrosis of appendages or rostrum with brownish black colouration are also indications of bacterial infection.
- Other symptoms include softening of cuticle, presence of spots and damage of the shell.

Characteristic colouration of the shrimps change during infections:

- Shrimps turn reddish when the hepatopancreas is affected due to toxic conditions or infected by a wide range of organisms.
- Colour of shrimps also changes due to specific diseases like yellow head virus infection with the change in the shrimp head colour to yellow.
- Mortality of Penaeus monodon has also been observed in certain cases associated with bluish colouration.
- Colour of the gills could give an indication of infection in shrimps.
- Presence of black gills point to bacterial infection and excessive white colour could be due to fungus and also could be due to gas bubble disease.
- Damage of gills could lead to respiratory failure in shrimps and eventual mortality.

Gill and appendage examination

- Remove a gill process and a mastigobranchial process (gill cleaner) and place it in a drop of clean seawater on a plain slide. Remove a labial palp and place it with the gill processes.
- Place the coverslip over the gills and examine with reduced light or phase contrast, beginning at low magnification.
- Parasites, fouling epicommensals and disease syndromes to look for include:
- Leucothrix mucor, filamentous blue-green algae, filamentous bacteria like Flexibacter spp. and possible fungal hyphae
- Protozoans; Zoothamnium spp., Epistylis sp., Acineta sp., Bodo sp., etc.
- Melanization of gill lamellae and / or gill cleaners due to a variety of necrotizing and inflammatory disease syndromes (histology usually required for confirmatory diagnosis).

Gastrointestinal examination

- Followed by external examination and gill observation, the gut examination has to be carried out. Following procedure is adopted.
- For examination of hepatopancreas, head is separated from abdomen to expose midgut and hepatopancreas and it is examined for atrophy
- Squash hepatopancreas between fingers and note colour and texture
- White and fluid filled indicates severe atrophy
- Presence of black streaks could indicate necrotising hepatopancreatitis (NHP), septic hepatopancreatitis (SHPNS, probable Vibrio sp. Infection), or aflatoxicosis
- Orange to reddish usually will be normal.

Microscopic examination:

- Remove HP and place it on a clean slide and bisect on mid line. Make impression smears and fix with methanol for 6 min and air-dry prior to staining. Giemsa staining is carried out as follows:

- ✓ Preparation of Giemsa stock solution - Mix 66.0 ml of glycerin and 1.0 g Giemsa powder.
- ✓ Place in 60 °C oven for 2 hours. Add 66.0 ml methyl alcohol and mix.
- ✓ Giemsa working solution – Mix 1.25 ml Giemsa solution, 1.50 ml methyl alcohol, 2 drops of sodium carbonate and add 50 ml distilled water.
- ✓ Preparation of Rosin alcohol stock solution – dissolve 10.0 g Rosin, white in 100.0 ml Absolute alcohol
- ✓ Rosin alcohol working solution – Mix Rosin stock solution 5.0 ml and 40.0 ml of 95% alcohol

- ✓ Stain the slide in working solution of Giemsa at room temperature for overnight or 30 min at 60 °C (use fresh solution, discard used solution)
- ✓ Differentiate in working rosin alcohol solution until smears assume a purplish pink colour.
- ✓ Dehydrate in absolute alcohol and clear in xylene and mount in DPX .
- ✓ Examine for colour, melanized foci or streaks, atrophy, and other signs that may be abnormal

- Remove with forceps several medial HP tubules and place in a drop of clean seawater on slide.
- If BP, MBV, or BMN are suspected, add small drop of 0.01% malachite green, squash with coverslip, and examine several items over next 5 min for baculovirus occlusion bodies and / or hypertrophied HP cell nuclei (as described earlier).
- If baculovirus is not suspected, coverslip the tissues and examine for lipid droplets (quantify grade of lipid droplets present. A few to none are sign of poor health and disease), melanized HP tubules, abnormally high numbers of motile rod shaped bacteria and gregarine trophozoites.

Gut examination

- Check the gut contents in midgut (MG) and stomach. For microscopic examination, dissect and remove MG from its junction with HP in head and hindgut in the sixth abdominal segment.

- ❖ Place MG on a dry clean glass slide with forceps and a coverslip on edge, strip MG to exude its contents onto slide.
- ❖ Add a drop of clean seawater to the contents, spread as needed, place coverslip and examine for,
- ✓ Gregarine trophozoites and/or gametocytes
- ✓ Baculovirus occlusion bodies (MBV or BP)
- ✓ Melanized masses of haemocytes that could indicate haemocytic enteritis or vibriosis

Examination of muscle or gonad for colour and texture

Examine for "cotton shrimp". This is due to microsporidian parasitism. Cramped muscle syndrome may result from a nutritional disorder in which affected shrimp display a flexure (cramp) of the abdomen that cannot be straightened. In severe cases, mortalities can be very high.

Microsporidian examination

Remove a suspect tissue from the shrimp and smear onto slide, add drop of clean seawater, and examine with phase of reduced light bright-field microscopy. Masses of uniform sized spores (visible at 200 to 400X) will be present if white muscle, gonad or HP disease was due to microsporidian infection.

Smear may be fixed and stained with Giemsa or an acid-fast stain to better display the spores.

Preservation of tissue samples

Samples required for histology, in situ hybridization, PCR or electron microscopy should be fixed on site by chemical preservation to prevent tissue breakdown and decay. For other techniques requiring live microorganisms like bacteriology, virology, mycology etc., the samples should be brought to the laboratory in ice with in 24 hours of sampling.

Collection of samples for histology

Collect the samples with minimum handling. Collect only live animals as far as possible. The samples are fixed or preserved in a fixative having 10 times volume that of the volume of the tissue. Common fixative used for shrimps is Davidson's fixative (Alcohol Formalin Acetic acid – AFA fixative)

Larvae and postlarvae (PL)

Immerse the shrimp directly into the fixative in case of larvae and postlarvae (PL). Fix for 12 to 24 h in a minimum of 10 volumes of fixative to one volume of shrimp tissue for effective preservation. Then transfer to 70 % alcohol for storage.

For PL that are more than 20 mm in length, make a small and shallow incision in the back using a fine needle and slightly lift the cuticle in the midline at the cuticular junction between first abdominal segment and cephalothorax. This is needed for quick and proper penetration of the fixative into the hepatopancreas.

Fixation of juveniles and adults

If possible, place the shrimp briefly in ice water for sedation. Fixation is done by injecting the fixative into the body of the live shrimp, using a needle and syringe. Depending on the size of the shrimp, 0.1 ml to 10 ml of fixative is injected (approximately 10% of the shrimp's body weight). The site of injection should be

anterior and posterior abdomen, anterior hepatopancreas and proper hepatopancreas. The shrimp should be properly fixed indicated by the change of colour. After the injection, slit open the shell. If the shrimp is larger than 12 g, bisect it at the junction of abdomen and cephalothorax. Immerse the shrimp in the remaining fixative. After 24-72 h and proper fixation, the specimen should be transferred to 70% ethyl alcohol. For transportation, remove the specimen from alcohol and wrap it in paper towels. Place the wrapped specimen in a sealable plastic bag and saturate it with 70% alcohol. Write complete history of the specimen and place the bag in a second sealable plastic bag. Many small sealable bags can be kept in one large sealable bag. This can be further transported to lab for processing.

Samples for PCR analysis

Samples for PCR analysis can be transported on ice and stored in -200c or should be fixed in either 95% or 70% ethanol. It is best to use cold fixative that has been stored in the freezer or kept on ice, as this helps arrest autolysis and secondary microbial proliferation, as the tissues are preserved. Samples can be gills, pleopods or pereiopods which could be chopped using a sterile scissors and put in the fixative.

Obtaining samples for microbiological diagnosis from shrimp

Nauplii, Larvae, PL

- ✓ Use the whole animal after rinsing in sterile seawater or 2.5% NaCl saline. Put the animal to broth culture medium and incubate.
- ✓ Place whole animal on agar medium surface, crush with loop, streak plate with exudate and incubate
- ✓ If transport to distant diagnostic lab is required, place sample in small sterile vial with sterile seawater, cover with sterile mineral oil. Keep transport sample vial cool, but do not freeze, as Vibrio species are sensitive to cold. Upon receipt at lab, transfer specimens to media.

Juveniles

- ✓ Disinfect the surface by dipping in 1% calcium hypochlorite or sodium hypochlorite for 10 to 60 second.
- ✓ Rinse in sterile seawater or 2.5% NaCl saline.
- ✓ Using alcohol flamed dissecting tools, remove the cuticle of an abdominal segment or of the carapace, excise the organ or a sample tissue of interest.
- ✓ For systemic infections, excise a block of abdominal muscle or the heart, touch it to the surface of an agar plate, streak and incubate.
- ✓ For enteric infections, excise the HP, midgut, fore gut and touch the exposed inner surfaces of the excised organ to the surface of an agar plate. Streak and incubate.

Animals large enough to bleed

- ✓ Remove haemolymph with a sterile tuberculin syringe and needle. A different syringe and needle must be taken for each set of shrimp. Place a drop of the haemolymph on an agar plate and inoculate with a sterile loop.
- ✓ An alternate method is to disinfect the antennae with alcohol then cut them. Place the drop of haemolymph appearing on an agar plate and streak with a sterile loop.

Culture media for initial isolation

- ✓ Marine agar (Zobell marine agar): A common medium used for bacteriological examination of marine bacteria.
- ✓ TCBS agar (selective agar): for Vibrio species
- ✓ Trypticase soy agar TSA with added NaCl 2%: can be used as both an isolation and purification medium
- ✓ Isolation
- ✓ Using aseptic technique, inoculate appropriate isolation agar(s) with sample to be tested.
- ✓ Each sample should be on a separate plate to avoid cross contamination
- ✓ Incubate the agar plates for 24h at room temperature or 25 to 300 C
- ✓ Check the plates at 12 to 18 h for luminescent colonies as the luminescence may fade quickly by 24 h.
- ✓ The organism should be purified on a general medium and further identified using staining, morphology, motility and biochemical tests.

Stuart transport medium

Stuart's transport medium can be used for transporting samples to distant laboratories. The composition of the medium is given below:

Sodium chloride - 3 g
Potassium chloride - 0.2 g
Disodium phosphate - 1.15 g
Monopotasium phosphate - 0.2 g
Sodium thioglycollate -1g
Calcium chloride 1% aqueous -10 g
Magnesium chloride 1% aqueous -10 g
Agar -4 g
Distilled water - 1000 ml
pH -7.3

The medium is essentially a solution of buffers, with carbohydrates, peptones, and other nutrients and growth factors excluded, designed to preserve the viability of bacteria during transport without significant multiplication of the microorganisms. Sodium thioglycollate is added as a reducing agent to improve recovery of anaerobic bacteria, and the small amount of agar provides a semi solid consistency to prevent oxygenation and spillage during transport. Sterile cotton swabs can be used for collecting the pathogens from any specific sites in the body of the fish after cleaning the surface with a disinfectant or chlorinated water and then wash with sterile saline. The ulcers or lesions could be swabbed and placed in the tube containing sterile medium. In case of small fishes they can be put directly into the medium after washing in sterile saline.

Video Link: *https://youtu.be/VPesqSyiSWQ*
https://youtu.be/4TdajCa7guc
https://youtu.be/unMkWTa17NI

Chapter 8
Pathology of Organ System of Finfish

Fish Dissection

For postmortem analysis of a healthy or diseased fish, dissection is one of the most important act.

Objectives

The objectives for that particular practical are to create a dynamic hands-on experience to assist in understanding the internal organs and system functions of a local fish species; for students to understand the external body parts of fish; and to discuss both internal and external features.

After this presentation, students will be able to:

- Identify 3-5 external anatomical features of a fish.
- Identify the major internal organs of a fish.
- Compare healthy and diseased fish internal organs and structures.

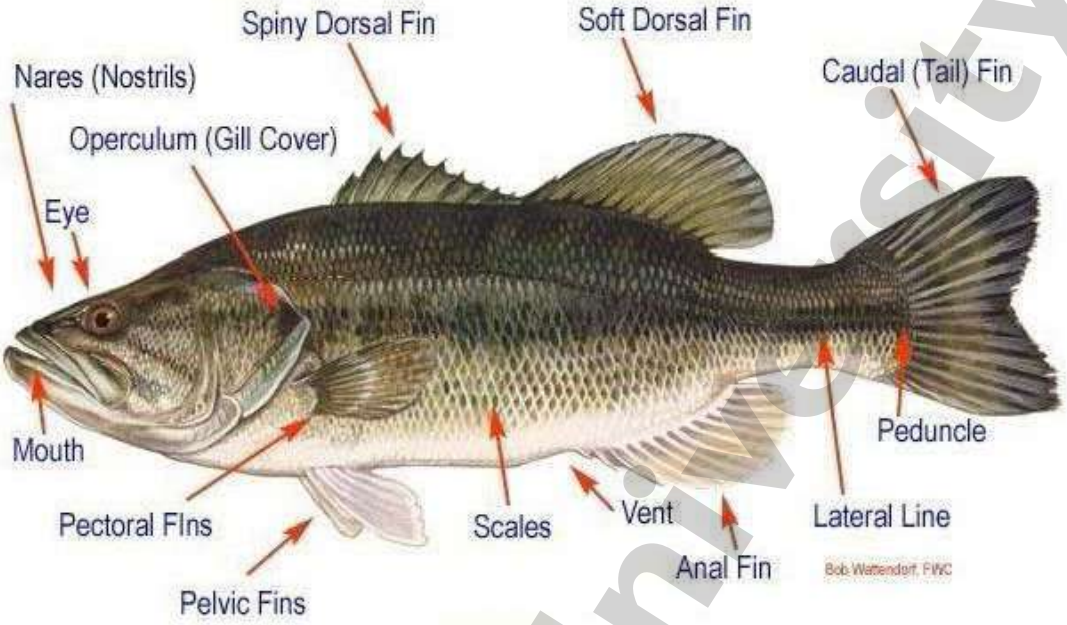
Materials

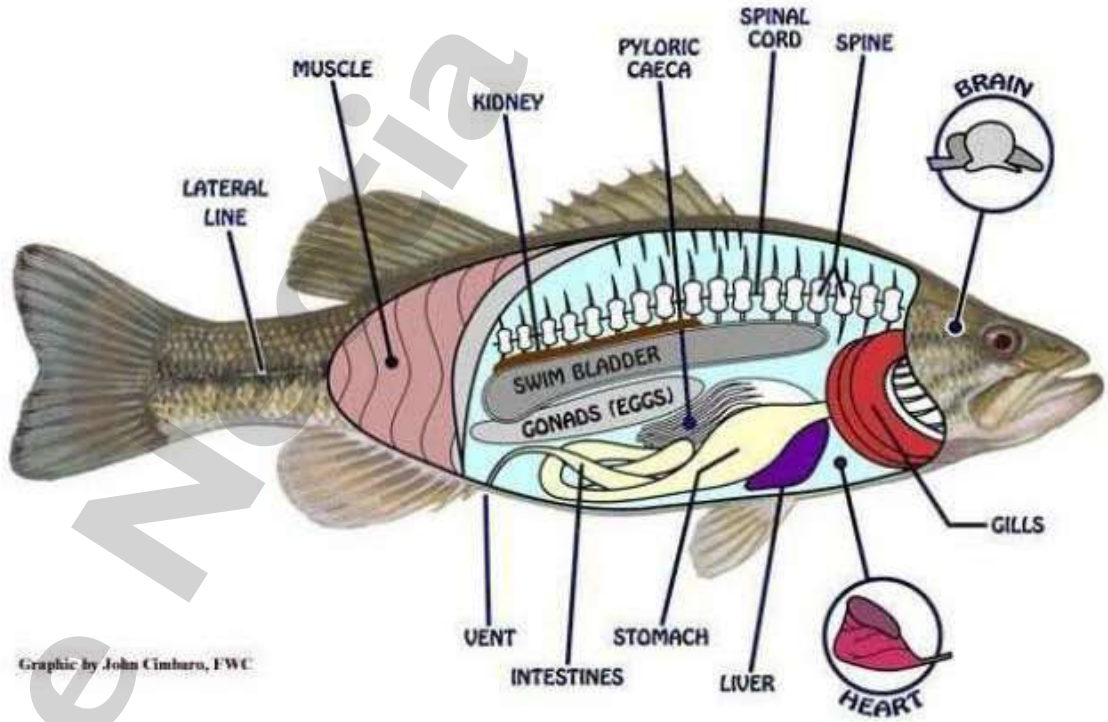
- Fish for dissection
- Scissors, scalpels, filet knives, or dissecting kits
- Dissecting trays or cutting boards
- Organ definition cards
- Hand sanitizer
- Laptop computer & projector
- Fish mounts, models or pictures
- Newspaper or protective table cover, 2 or more rags
- Box of plastic gloves (non-latex)
- Dissection Power Point presentation
- Dissection Worksheet

Methods

Start by finding the anus (the only opening on the underside), and cutting a line not too deep all the way up to the gills. Open it up a bit, and then cut a lateral line up the side of the fish so you can open it up to see the way the guts are inside while they're in place.

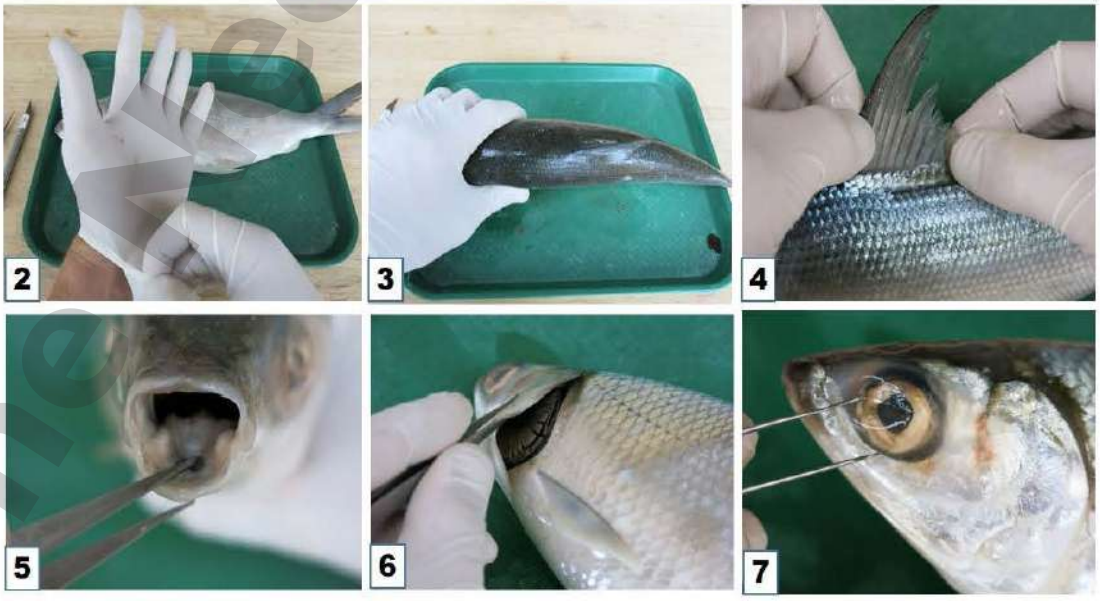
Internal and External Anatomy of Fish :

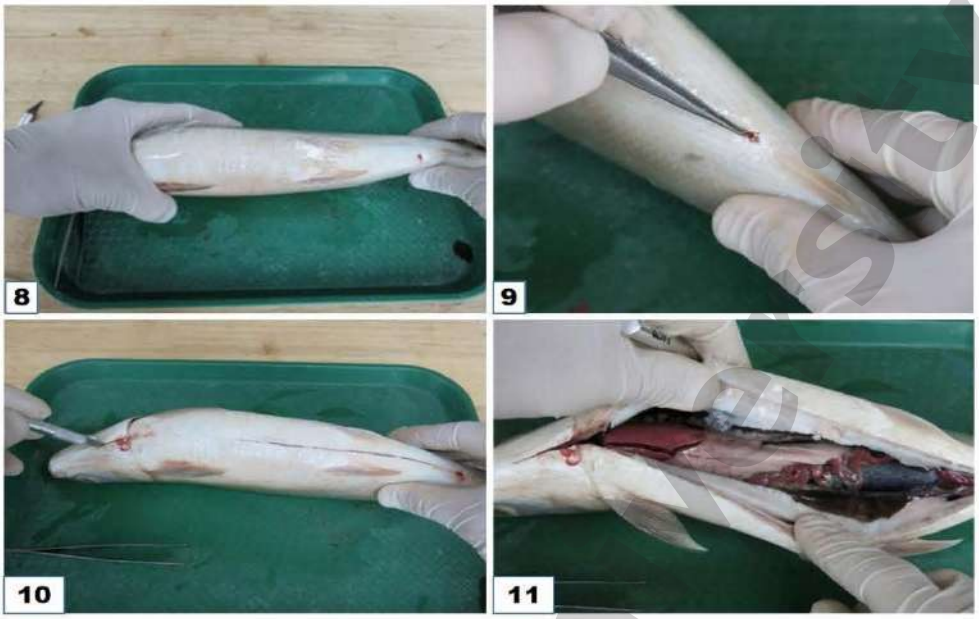




DISSECTION BOX

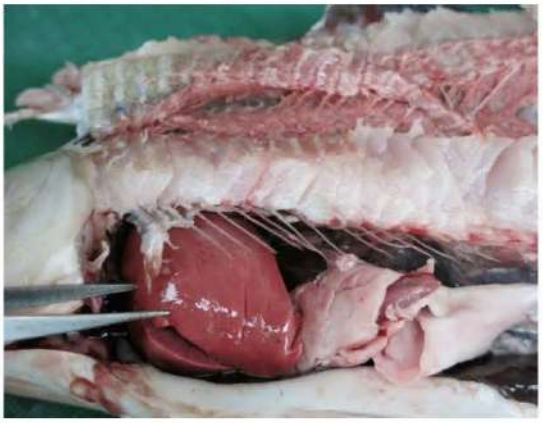
How to dissect a fish ?





Digestive System

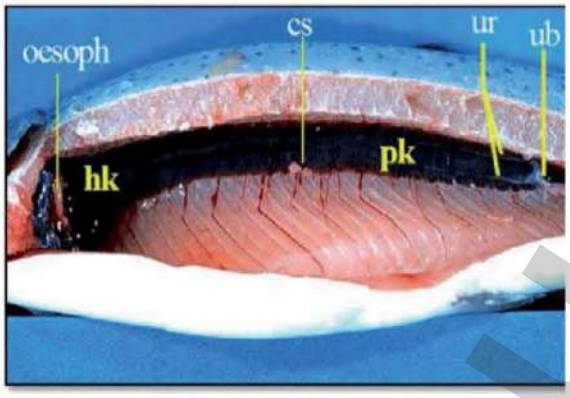
Heart



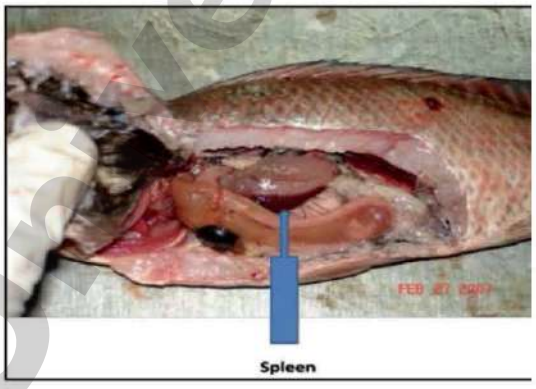
Liver

Swim Bladder

Gill



Head Kidney (hk), Posterior Kidney (pk), Corpuscle of stannous (CS), Ureter (ur) and Urinary bladder (ub)



Spleen

Fish Dissection: https://youtu.be/Hzc6nApVGGE
https://youtu.be/jnSYB3FsNX8

Chapter 9

Pathology of Organ System of Shellfish

Fish Dissection

For postmortem analysis of a healthy or diseased fish, dissection is one of the most important act.

Objectives

The objectives for that particular practical are to create a dynamic hands-on experience to assist in understanding the internal organs and system functions of a fish species; for students to understand the external body parts of fish; and to discuss both internal and external features.

After this presentation, students will be able to:

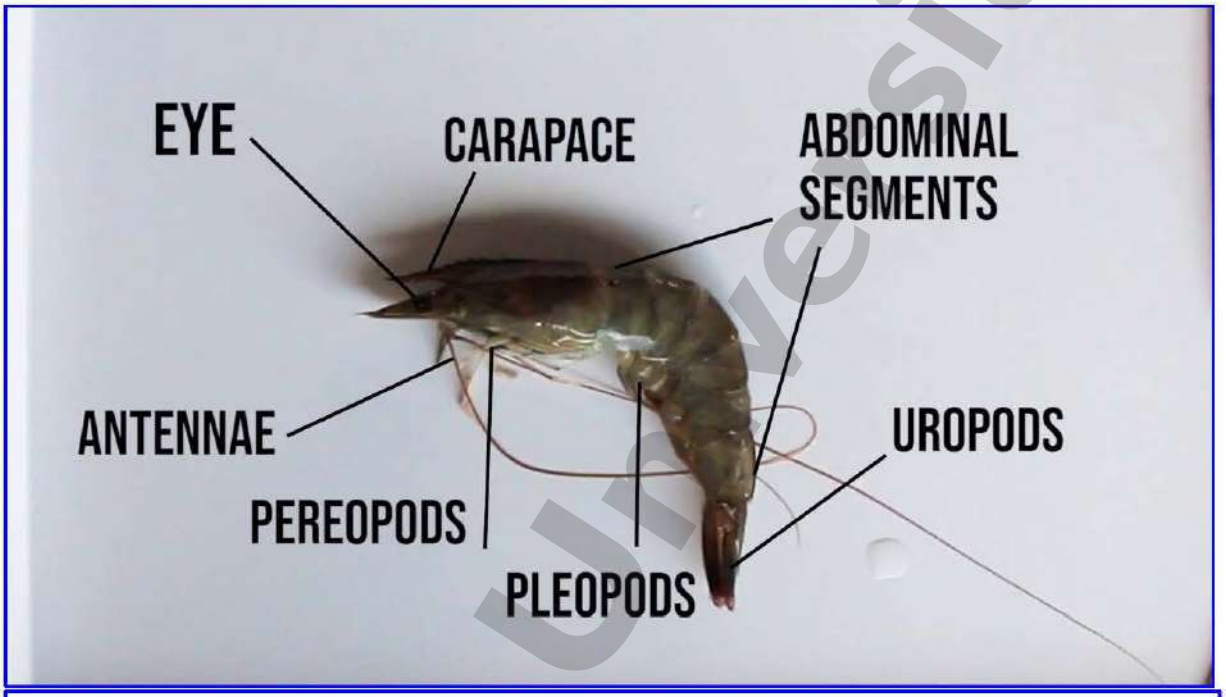
- Identify 3-5 external anatomical features of a fish.
- Identify the major internal organs of a fish.
- Compare healthy and diseased fish internal organs and structures.

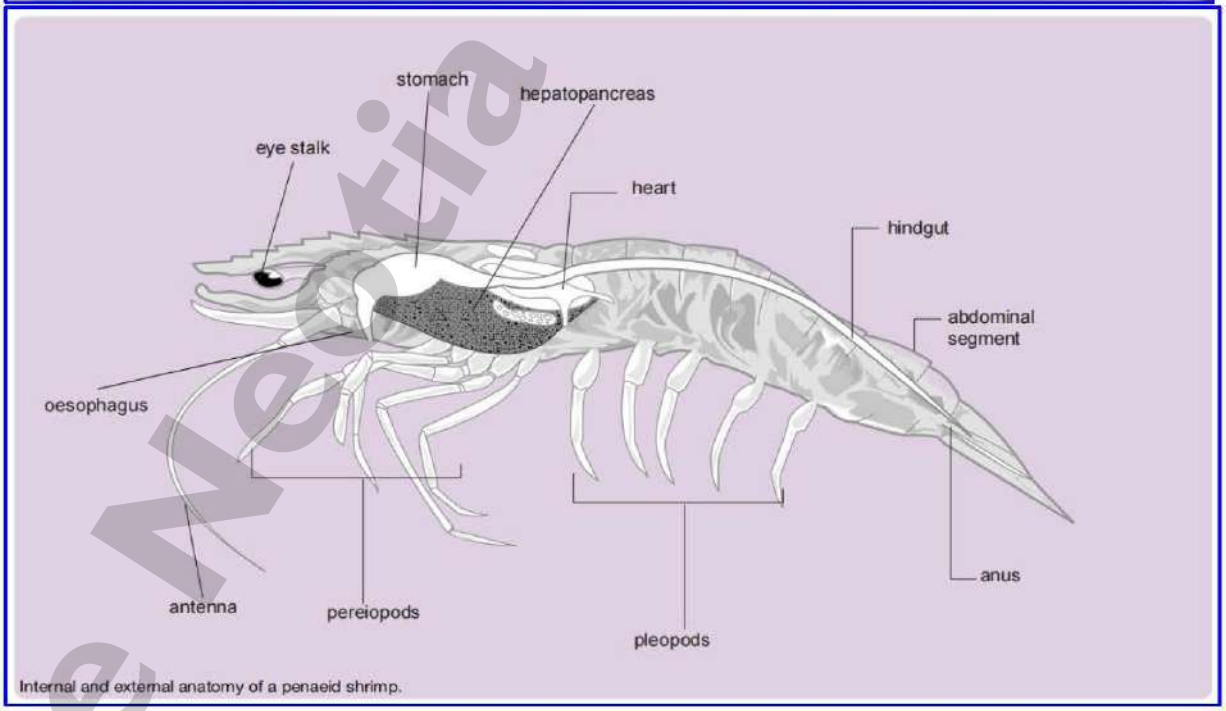
Materials

- Fish for dissection
- Scissors, scalpels, filet knives, or dissecting kits
- Dissecting trays or cutting boards
- Organ definition cards
- Hand sanitizer
- Laptop computer & projector
- Fish mounts, models or pictures
- Newspaper or protective table cover, 2 or more rags
- Box of plastic gloves (non-latex)
- Dissection Power Point presentation
- Dissection Worksheet

Video link: https://youtu.be/2daMyqfd73E

External and Internal Anatomy of Shellfish:

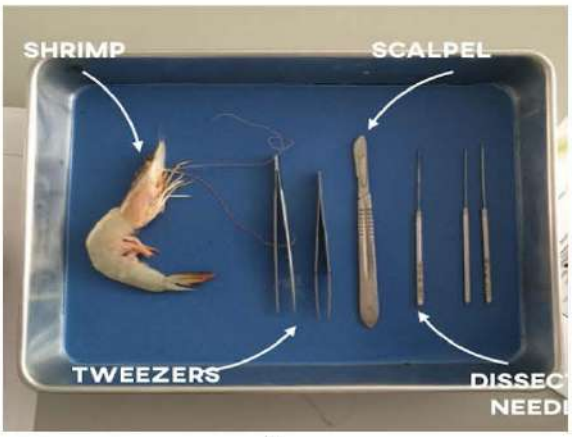




Internal and external anatomy of a penaeid shrimp.

Shrimp dissection: https://youtu.be/MDN3OM1TQAQ

How to dissect a shellfish (Shrimp) ?



1.

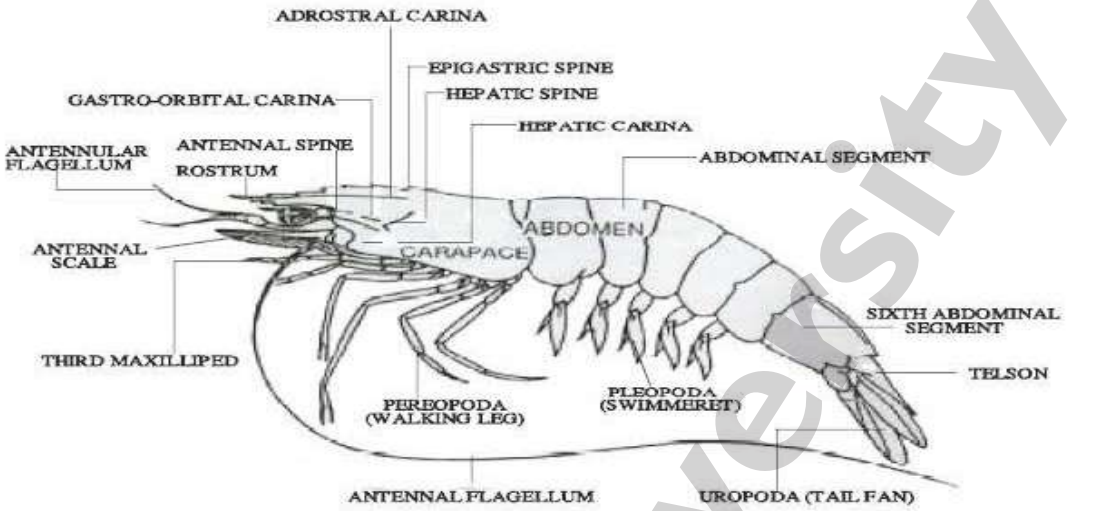
2. Cut the shrimp along the x axis from dorsal side. Try to cut without separate two bodies.

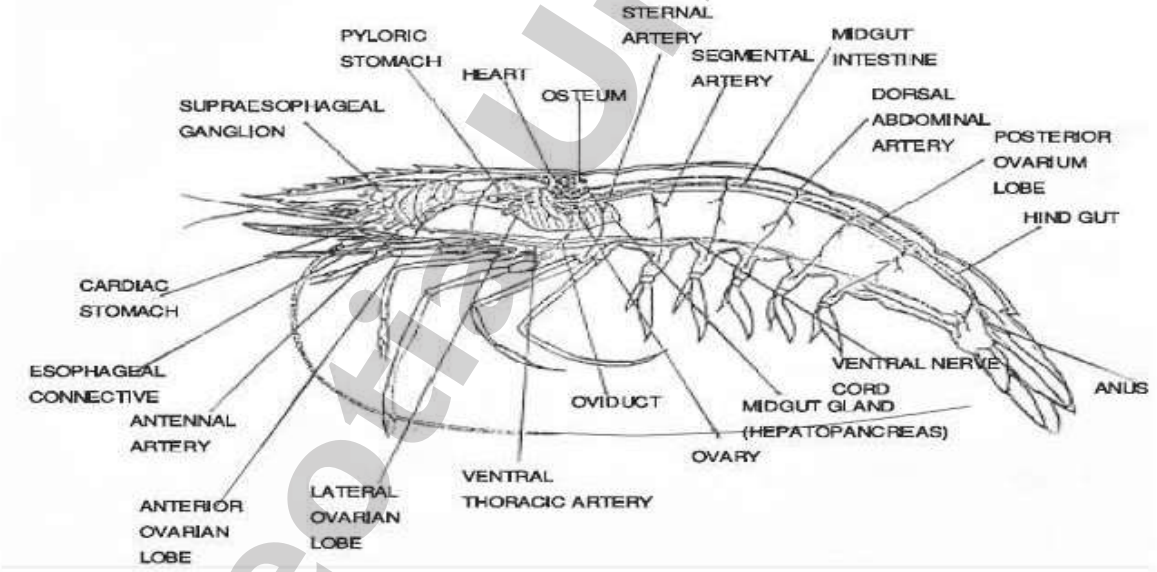
3. Use needle to attach shrimp with dissection board.

4. Observe a head part, we can see the antenna which is the long string in the head. Also, rostrum, eyes, brain, bladder, and carapace are included in head part.

5. In abdomen part, we see swimmerets, muscles, and digestive glands.

6. We observe uropod and telson the tail part.





Chapter 10
Histopathology Procedures

Histopathology guidelines for fish and shellfish

- ✓ Live (after anesthetizing)/ moribund fish should only be used
- ✓ Never freeze tissue meant for histology
- ✓ Tissue samples should be fixed with fixative in a wide mouth plastic bottle as early as possible after removal from water or time of death prior to transporting to the lab, since, fish rapidly autolyze
- ✓ In the case of larger fish (>6 cm), dissect-out tissue having preferable size of <1 cm from specified organ with sharp blade or scissors and transported in fixative
- ✓ In the case of small fish, the specimen is slit open/ inject the fixative into the intestinal cavity before immersing in fixative to ensure better penetration of fixative
- ✓ If the specimen is a larger shrimp (>12g), inject the fixative at 3 or 4 places (cephalothorax, anterior abdomen and posterior abdomen) and put in the fixative for 48 hours

(Note: The cuticle should be slit open on both sides of the shrimp from the sixth abdominal segment to the rostrum using scissors, before placing in fixative to ensure the proper penetration of fixative. Organs are dissected out and fixed separately. Hepatopancreas should be immediately fixed post death.)

- ✓ In case of post larvae (PL) and small shrimp (<12 g), directly immerse the specimen in fixative for 24 hours
- ✓ The volume of fixative must be at least 10 times the volume of the tissue; The fixative must be replaced after 24hours
- ✓ The commonly used fixatives are 10% neutral buffered formalin (NBF) for fish and Davidson's fixative for shellfish
- ✓ Formalin based fixatives are hazardous, so skin and eye contact/ inhalation should be avoided
- ✓ Fixatives in alcohol should be specifically noted on the container during transport due to their inflammable nature
- ✓ Seal the container properly
- ✓ Details of sample should be labelled on paper and placed inside the fixative container (labelling should be made with pencil, as alcohol and fixatives tend to wash off pen marks on the container)
- ✓ Samples put in Bouin's fluid or Davidson's fixative should be transferred to 70% alcohol after 24-48 hours
- ✓ Conduct histological analysis as per standard procedures.

Procedures

1. Organs collection

1.1 In live animal procedures, obey the recommendations of the Ethics Committee on Animal Use. Samples should be collected on freshly dead fish, not frozen or "Extremely Dead"

1.2 Select the appropriate tweezers, scissors and scalpels to remove the organs for analysis, so that the tissues are not damaged at the extraction.

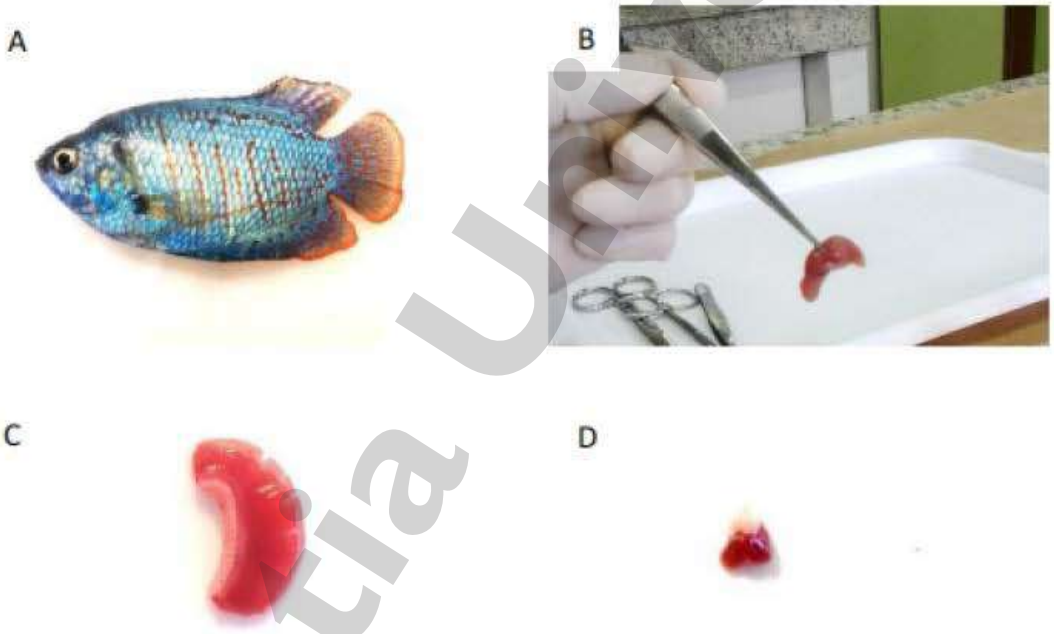
1.3 The main organs of choice for performing histological analysis are gill, liver, spleen, kidney, stomach, intestine, heart, gonad and skin. Sample volume should not exceed 1/10th of the volume of fixative.

- ❖ Fry can be fixed as whole.
- ❖ Fingerlings and smaller fish :

–Gill opercula cut off
–Cut along midline
–Viscera pulled out

- ❖ Larger Fish

–Take samples in field
–Samples should be no thicker than 3mm

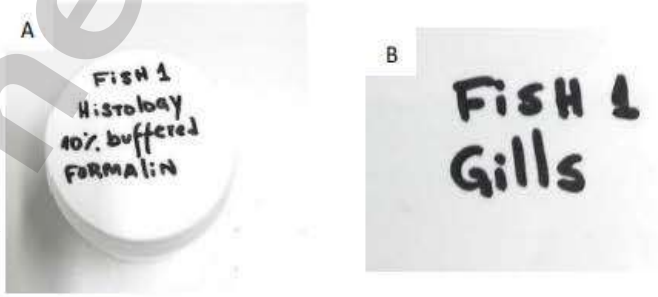


Standard procedure for removal of organs for histological analysis. A – Colisa *Trichogaster* sp.; B – Liver sample; C – Gill sample; D – Heart sample.

2. Organs fixation

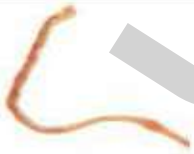
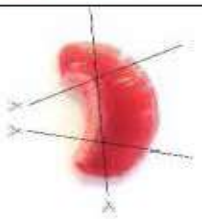
2.1 After removing the organs, proceed to fixation in 10% buffered formalin, contained in previously identified vials. For gonads, the fixation in Bouin's solution is recommended.

2.2 The organs should remain in the fixative for 24 to 48 hrs after collection and, thereafter, should be transferred to 70% alcohol until the processing time.



A – Identification of the sample and the type of fixative on the vial lid where the organs will be stored; B – Identification label of the sample written on butter paper to place inside the vial.

Table 1 Cutting procedures of organs for histology

HEART		
LIVER		
SPLEEN		
KIDNEY		
INTESTINE		
STOMACH		
GILL		

3. Organs processing

3.1 Make a double identification on cassettes: an external inscription in pencil and an internal stamp made with butter paper also written in pencil.
3.2 Remove the organ from the vial containing alcohol 70% and place it on a petri dish previously prepared with dental wax immersed in alcohol 70%.
3.3 With a scalpel blade, under stereomicroscope, make the cutting procedure of the organs as shown in Table 1.
3.4 Place the organs inside the cassette and close it.
3.5 Keep the cassette inside a beaker containing alcohol 70% until the next step (dehydration of the organs).

A – Materials for the organ cleavage B - Cassettes with external identifications; C - Separation of organs in the Petri dish with dental wax; D - Cassettes with the organs stored in alcohol 70%.

4. Dehydration, diaphanization and organs impregnation

4.1 Transfer the cassettes stored in the beaker containing alcohol 70% to the cassette holder of the automatic tissue processor.

4.2 Dehydration of the organs allows the exchange of the water of the tissue for alcohol; the diaphanization promotes the substitution of alcohol from the sample with xylol; and the impregnation consists of replacing xylol with paraffin (Table 2).

Table 2 Organ dehydration scheme for histology

REAGENT	TIME
1 – Alcohol 70%	1 hour
2 – Alcohol 70%	1 hour
3 – Alcohol 95%	1 hour
4 – Alcohol 95%	1 hour
5 – Alcohol 100%	1 hour
6 – Alcohol 100%	1 hour
7 – Xylol	1 hour
8 – Xylol	1 hour
9 – Paraffin	65°C (149°F) for 1 hour
10 – Paraffin	65°C (149°F) for 1 hour
Observation	After step 10, immediately start embedding.

Automatic tissue processor

P.S.: Always use Personal Protective Equipment (mask, nitrile gloves, lab coat and safety goggles).

5. Organs embedding

5.1 Turn on the automatic embedding machine to heat the filtered paraffin (65°C = 149°F)

5.2 Prepare the stainless base molds to receive the organs

5.3 Place the stainless base mold below the dispenser and fill it with paraffin

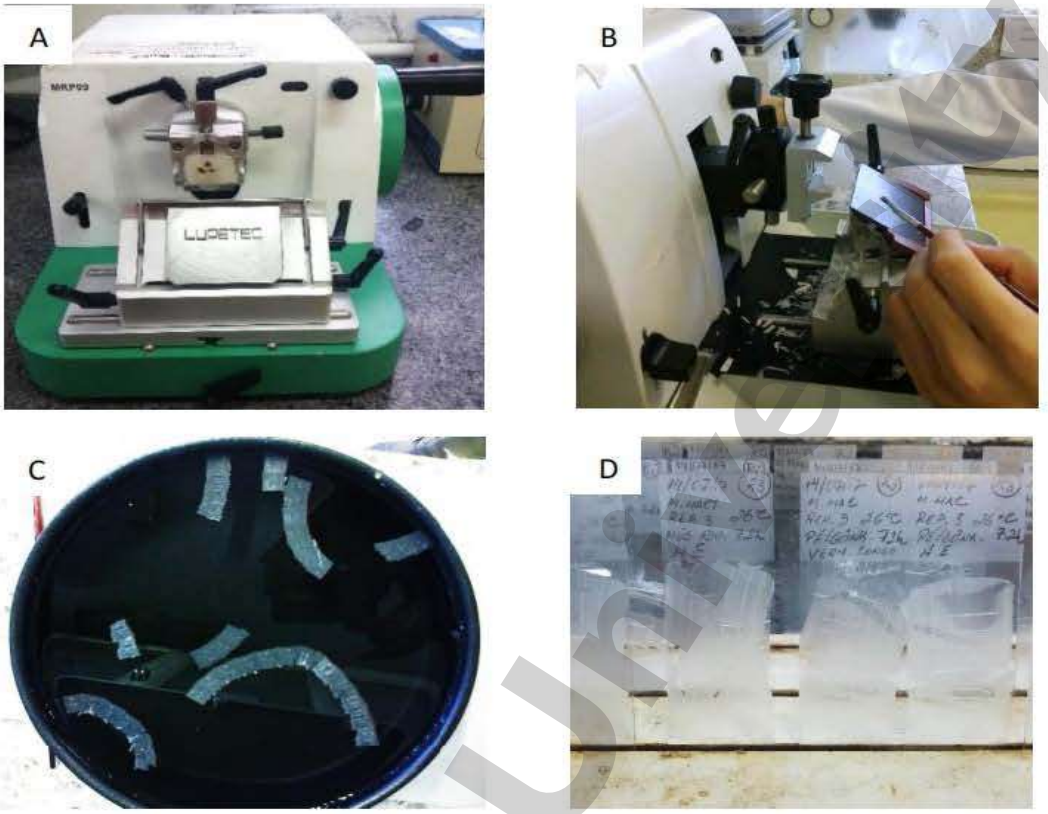
5.4 Remove the organs from the cassette and arrange them, with the aid of heated tweezers, into the stainless base bold

5.5 Let the stainless base mold cool to room temperature for 24 hours and, after the paraffin solidifies, unmold the blocks.

A – Automatic embedding machine; B – Stainless base mold; C – Paraffin dispenser; D - Paraffin block with organs ready to be cut.

6. Histological sections

6.1 Leave the blocks in the freezer at least 24 hs before cutting
6.2 Place the block on the microtome block holder, and adjust the cutting scale (3 to 4 mm) and then thin the paraffin until all organs are in the same level
6.3 Begin cutting the block to the selected thickness until the paraffin ribbon containing the organs is formed
6.4 Transfer the ribbon into a warm water bath at 45°C (113°F) so that it can be stretched, avoiding overlapping and rupturing points in the organ
6.5 Once stretched, the ribbon should be scooped up onto a previously cleaned slide After making the slide (in duplicate), let it rest in a drying oven at 30°C (86°F).



A - Microtome for semi-thin cuts; B – Making ribbons by thin sections; C – Ribbons stretching in warm water bath; D - Identified slides containing the paraffin ribbon with the organs.

7. Staining and permanent slides

7.1 In the Hematoxylin-Eosin (H-E) staining process, the slide containing the paraffin ribbon and organs should be transferred following the sequence of steps 1 to 19 (Table 3)
7.2 In some cases, the times listed on table 3 can vary depending on the tissue; so, it is recommended that you run tests with some duplicate slides before processing all other slides
7.3 At the end of the process, the permanent slide should be made by gluing a cover glass over the stained slide
7.4 Depending on the purpose of the research and on what to look for, other staining may be necessar.y

Examples include the following:
Prussian blue, by the Pearl method, it is used to evaluate iron deposits of biological origin in organs such as spleen and kidneys; with **Masson's trichrome** it is possible to identify collagen fibers; with **Gomori trichrome** is evidenced fibrosis (muscle fibers) and collagen; the **Giemsa stain** (Romanowsky) allows the identification of microorganisms in general, such as parasites, bacteria and even fungi.

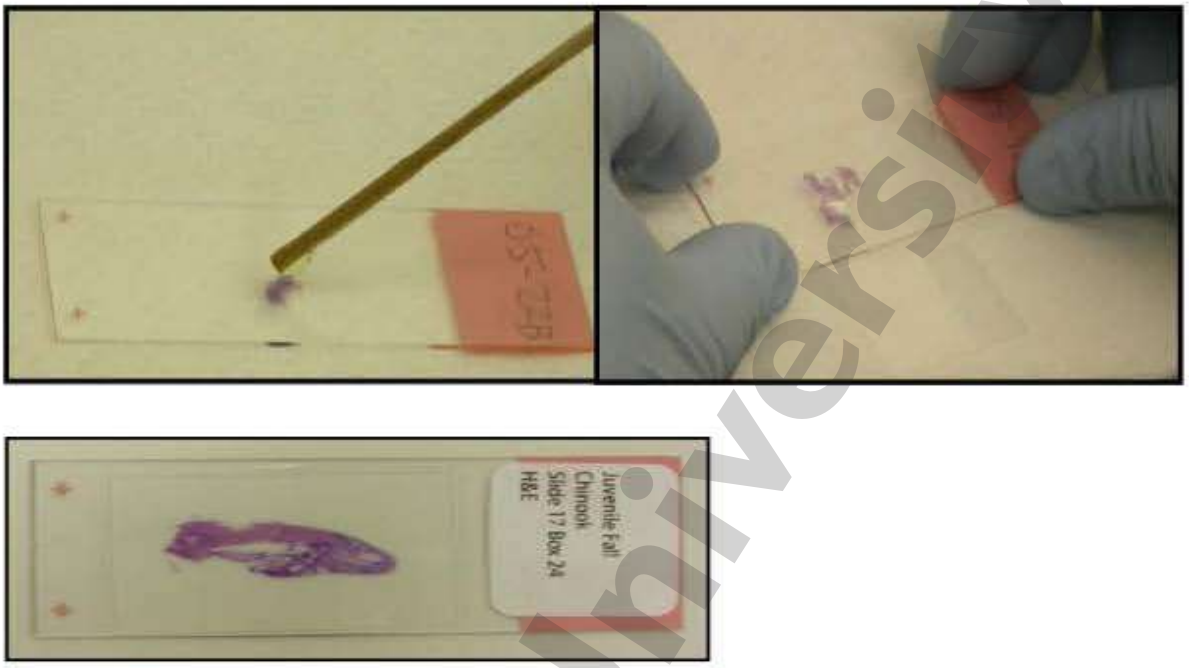
Table 3 Procedures for histological slide staining with Hematoxylin and Eosin (H&E)

STEP	TIME/OBSERVATION
1 – Xylol	4 minutes
2 – Xylol	4 minutes
3 – Alcohol 100%	4 minutes
4 – Alcohol 100%	4 minutes
5 – Alcohol 90%	4 minutes
6 – Alcohol 80%	4 minutes
7 – Alcohol 70%	4 minutes
8 – Running tap water	5 minutes
9 – Hematoxylin	2 minutes*
10 – Running tap water	10 minutes
11 – Distilled water	3 immersions and quick suspensions **
12 – Alcohol 70%	3 immersions and quick suspensions**
13 – Eosin Y solution	11 minutes
14 – Alcohol 95%	3 immersions and quick suspensions **
15 – Alcohol 100%	4 minutes
16 – Alcohol 100%	4 minutes
17 – Alcohol + Xylol	4 minutes
18 – Xylol	4 minutes
19 – Xylol	Until the moment of the slide assembly

***Suspend when the timer marks half the time (1 minute) and immerse it again to complete the total time; ** Suspend the rack and immerse it again three times.**

Staining

Coverslipping



Analysis and interpretation of tissue

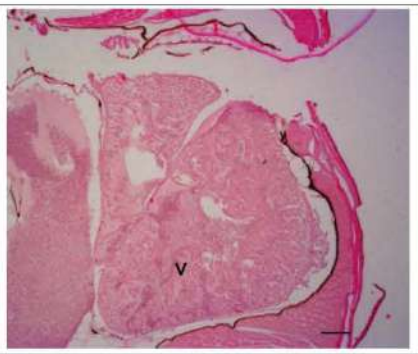
alterations For analysis and interpretation of the alterations, you must have knowledge about how the configuration of healthy tissue is; only then it will be possible to make comparisons and identify possible abnormalities at cellular and tissue levels. Therefore, it is recommended a prior consultation in bibliographical materials such as articles and books, not only to understand the functioning of organs, but also to understand their anatomy, and how they are physically structured: type, shape and arrangement of cells, presence of vessels and arrangement of muscle fibers, just to name a few examples. When you start to reading histological slides, it is suggested to prepare a table (see Appendix) containing a list of possible alterations for each specific organ; in it, the changes can be checked qualitatively and quantitatively, as follows: F (focal) to alteration in a specific point of the organ, M (multifocal) for alterations in more than one point of the organ e C (coalescent) for alterations that affect the entire organ, 0 for absence of lesion, 1 for lesions that compromise up to 25% of the organ, 2 for commitment of up to 50% e 3 for alterations that affect more than 50% of the organ.

Video Link: https://youtu.be/VFxWpNDrgYE
https://youtu.be/jX88IGSLqvA
https://youtu.be/7-LIbAWPc-g

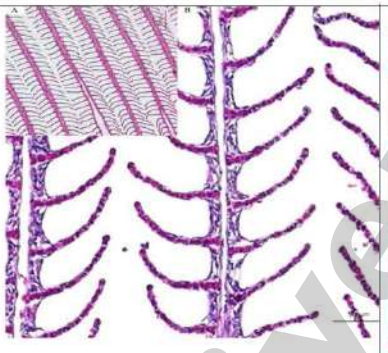
Chapter 11

Histopathology of Finfish

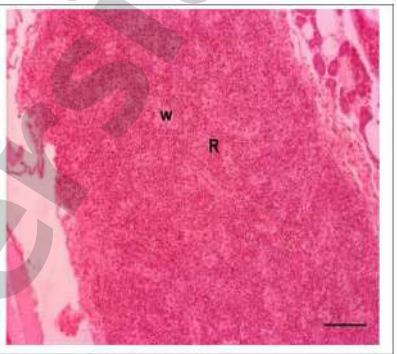
Histopathology of healthy fish



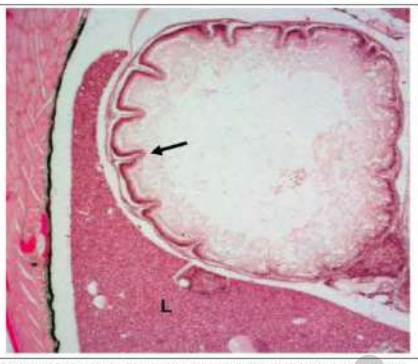
Heart parenchyma showing arrangement of cardiac muscles; (V) ventricle.



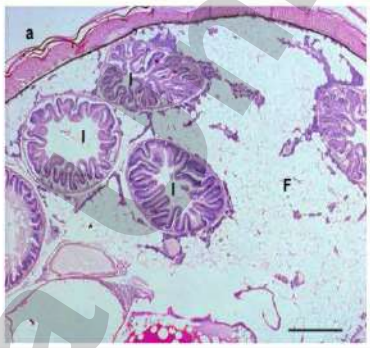
Gills A – Broad view of healthy gill; B – Close view of healthy gill.

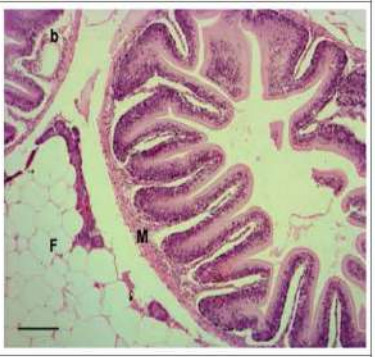


Sspleen with red (R) and white (W) pulp.

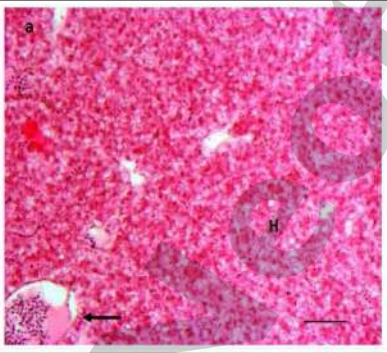


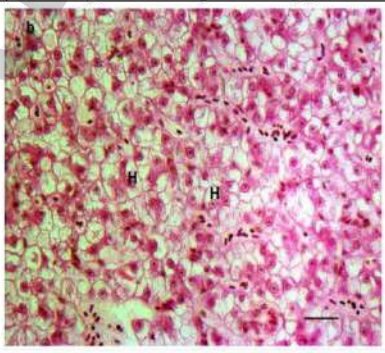
(S) stomach featuring short microvilli (arrow). (L) liver.



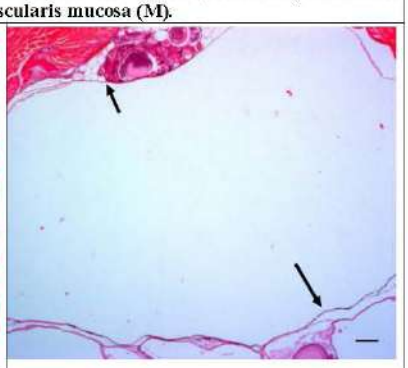


a) (I) Small intestine - transverse section; Abdominal adipose (fatty; F) tissue in the body cavity, surrounding the intestine; b) The mucosa can be seen, including microvilli (arrows), columnar epithelium (E) and muscularis mucosa (M).

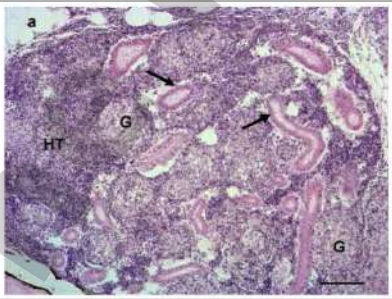


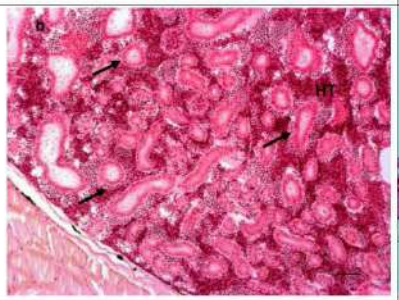


Liver tissue composed of hepatocytes (H); blood vessels (arrow).

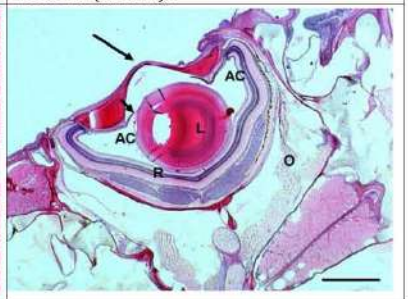


Abdominal cavity showing inflated swim bladder (arrows).

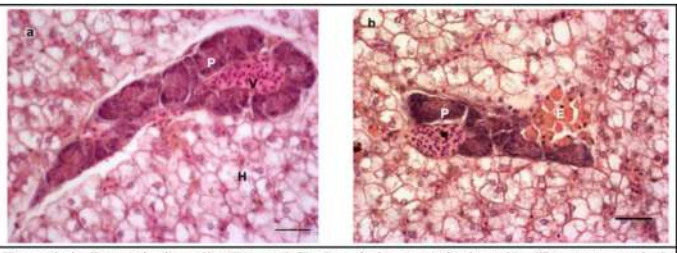




a) Head kidney showing renal tubules (arrows), hematopoetic tissue (HT) and renal corpuscle with its glomeruli (G); Bar=10 µm. b) Trunk kidney showing more renal tubules (arrows) and little amount of hematopoietic tissue (HT).

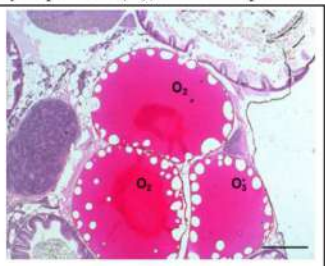


Eye showing: lens (L), retina (R), aqueous cavity (AC); iris (short arrow); cornea (long arrow); and optic nerve (O).

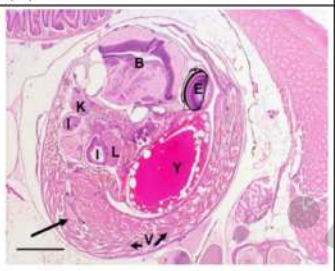


Exocrinic Pancriatic cells (P) and Endocrinic pancriatic cells (E) surrawnded by hepat cells (H); branch of portal vein (V).

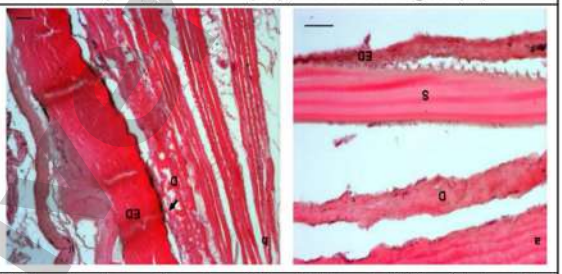
Brain showing olfactory lobe (OL); optic lob (OpL); cerebellum (Cer); cerebrum (Cr); Medula oblongata (M).



Female gonads showing oocytes in different stages of development (O2- stage IV, O3- stage V).



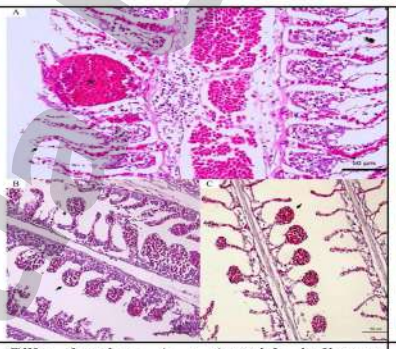
Guppy's Fetus showing: yolk sac (Y); vertebral column (V- short arrows); intestine (I); kidney (K); liver (L); spinal cord (arrow); brain (B).



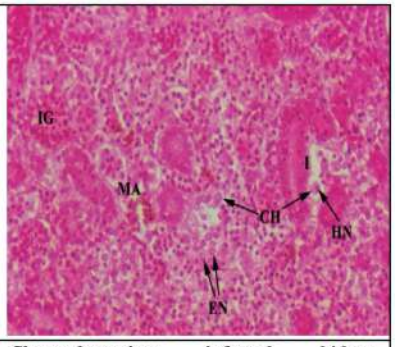
Skin showing: Dermis (D); Epidermis(ED), Scale (S); Chromatophores (melanocytes) (arrow).

Histopathology of diseased fish

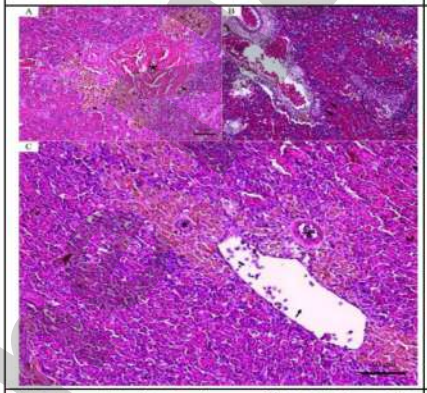
Gills showing: A – Broad view of coalescent interlamellar hyperplasia; B – Arrow indicates hyperplasia of secondary lamellae; C – Arrow indicates on a close view a interlamellar hyperplasia with almost total fusion of secondary lamellae.



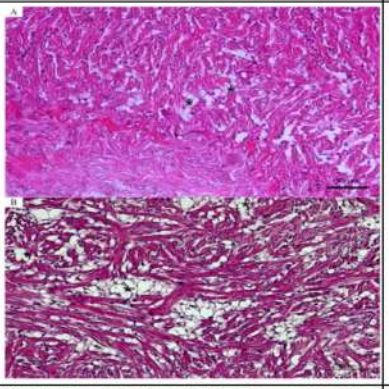
Gills showing: A - Asterisk indicates aneurysm and the arrow indicates a detachment of epithelium layer in the secondary lamella; B – Arrow indicates telangiectasia; C – Arrow indicates telangiectasia.



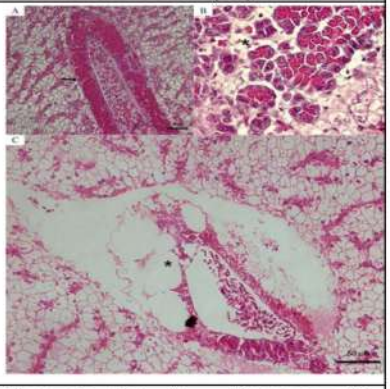
Chryseobacterium infected kidney showing: cellular hypertrophy (CH), nuclear hypertrophy (HN), inflammation of renal tubules (I), mild melanomacrophage aggregate (MA), inflamed glomerulus (IG), and epithelial cells devoid of a nucleus (EN).



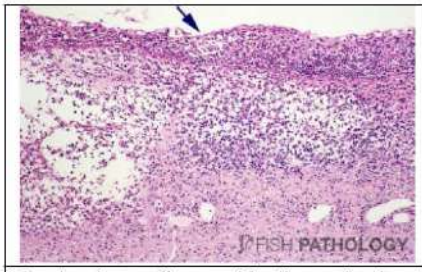
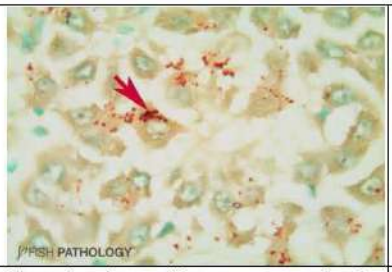
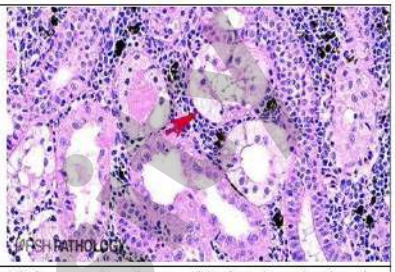
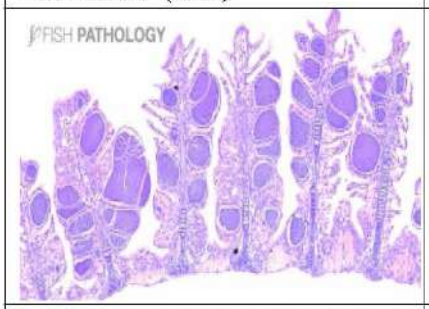
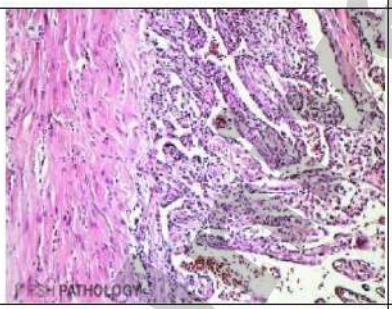
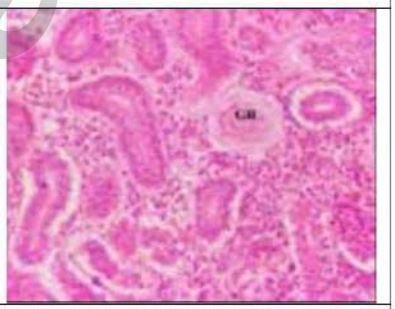
Spleen showing: A – Congested vein (asterisk); B – White pulp (circle) and red pulp (arrows); C – Arteriole (asterisks) and blood vein without congestion (arrow).



Heart showing: A and B – Severe cardiac steatosis inventricle (asterisks).



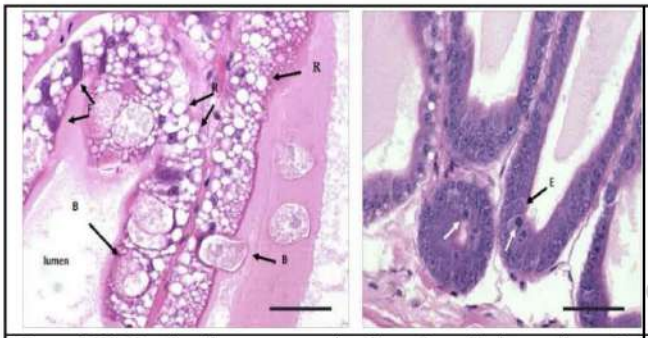
Liver showing: A – Pancreas with apparent eosinophilic zymogen granules (arrow); B and C - Loss of pancreas structure (asterisk).

		
In strawberry disease, skin tissue showing: severe inflammation, and loss of scales, the epidermis is involved to the same extent as the dermis, and there are many more neutrophils. Within the epidermis can be seen an aggregate of neutrophils, forming a "micro-abscess" (arrow).	Liver showing positive copper reaction in hepatic cytoplasm. Rhodamine stain. Note the intracytoplasmic granules (bright red or rust-red) which corresponds to lysosomes with sequestered excess copper (arrow).	Kidney showing: Moderate hydropic degeneration of the tubular epithelium due to copper toxicity (arrow).
		
Gill showing: abundant lamellar basophilic inclusions are observed, consistent with severe epitheliocystis infection.	Cardiomyopathy syndrome (CMS) in ventricle showing severe myocarditis of spongy layer. Note that the compact layer (on the left) is largely unaffected.	Flavobacterium infected kidney showing granuloma-like formation (GR).

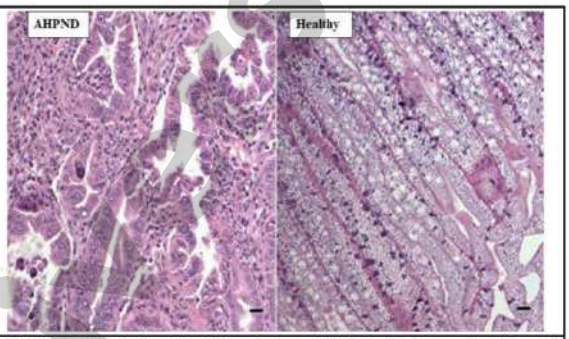
Video link: https://youtu.be/4y6lLHdi13o

Chapter 12
Histopathology of Shellfish

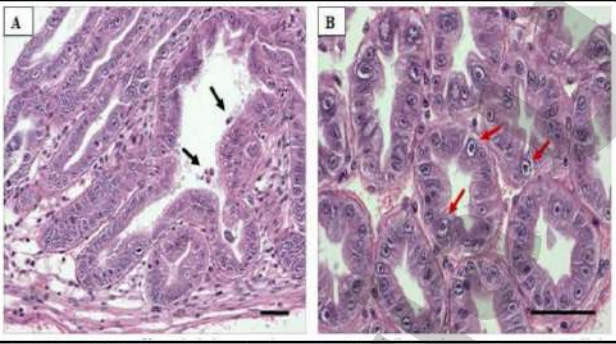
Histopathology of diseased shellfish



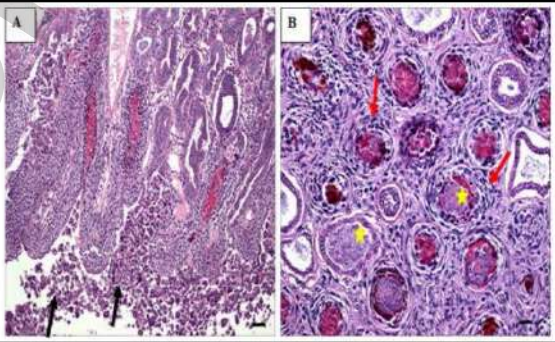
Normal HP showing the presence of embryonic or Embryozellen cells (E), fibrillar or Fibrillenzellen cells (F), resorptive/absorptive or Restzellen cells (R), and blister or Blastozellen cells (B) cells. White arrows: metaphase.



AHPND-affected vs healthy shrimp. Histological examination of the HP of *P. vannamei*. Left: early stage of diseased shrimp. Right: healthy shrimp.

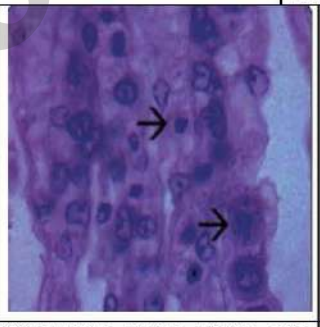


AHPND-affected shrimp. Early acute stage of diseased *P. vannamei*. (A) slight sloughing of epithelial cells (black arrows) examined at a low magnification, (B) the appearance of enlarged nuclei (karyomegaly, red arrows) under a higher magnification.

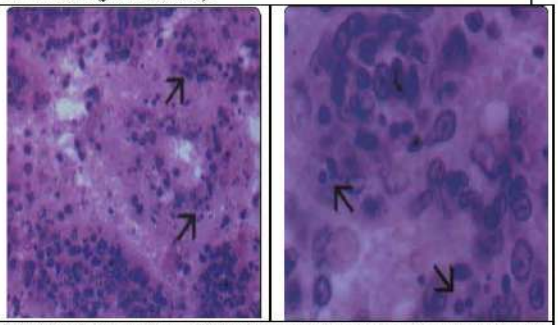


AHPND-affected *P. vannamei*, terminal phase. (A) Sloughing of tubule epithelium (black arrows), significant proximal haemocytic inflammation; most tubules are destroyed, some tubules with putative vibriosis. (B) Extensive haemocytic infiltration (red arrows), massive bacterial colonization in tubule lumens (yellow stars).

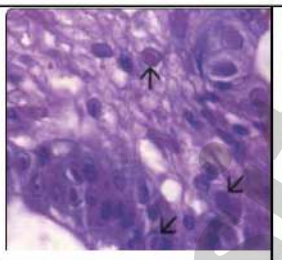
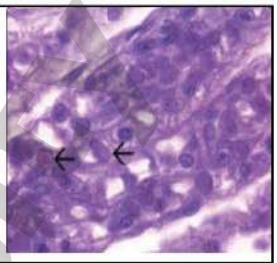
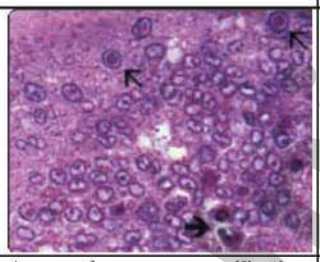
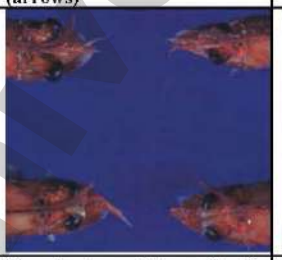
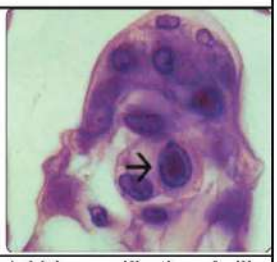
Gross sign of yellow head disease (YHD) are displayed by the three *Penaeus monodon* on the left.

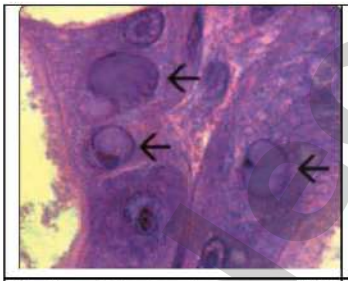
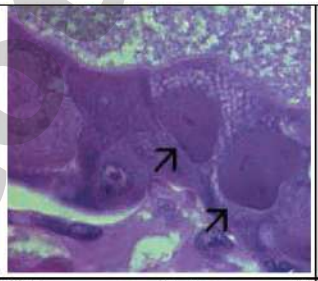
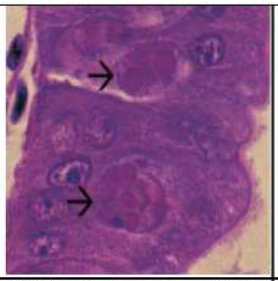
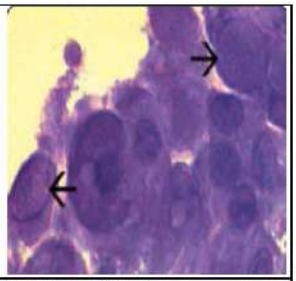


Histological section of the gills from a juvenile *P. monodon* with YHD. A generalized diffuse necrosis of cells in the gill lamellae is shown, and affected cells display pyknotic and karyorrhectic nuclei (arrows). A few large conspicuous, generally spherical cells with basophilic cytoplasm are present in the section.These cells may be immature hemocytes, released prematurely in response to a YHV-induced hemocytopenia.

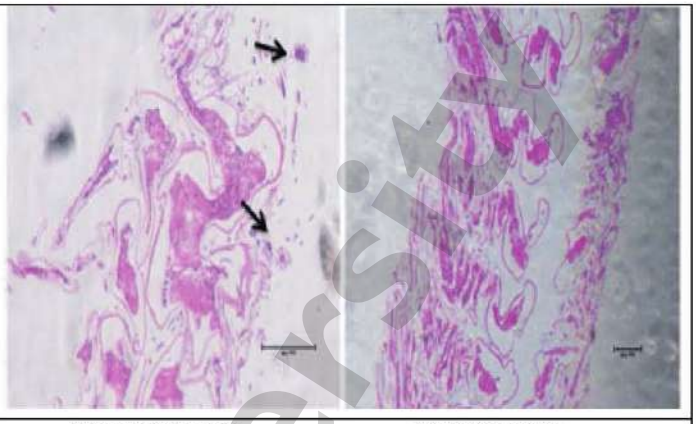


Histological section of the lym phoid organ of a juvenile *P. monodon* with severe acute YHD at low and high magnification. A generalized, diffuse necrosis of LO cells is shown. Affected cells display pyknotic and karyorrhectic nuclei. Single or multiple perinuclear inclusion bodies, that range from pale to darkly basophilic, are apparent in some affected cells (arrows). This marked necrosis in acute YHD distinguishes YHD from infections due to Taura syndrome virus,which produces similar cytopathology in other target tissues but not in the LO.

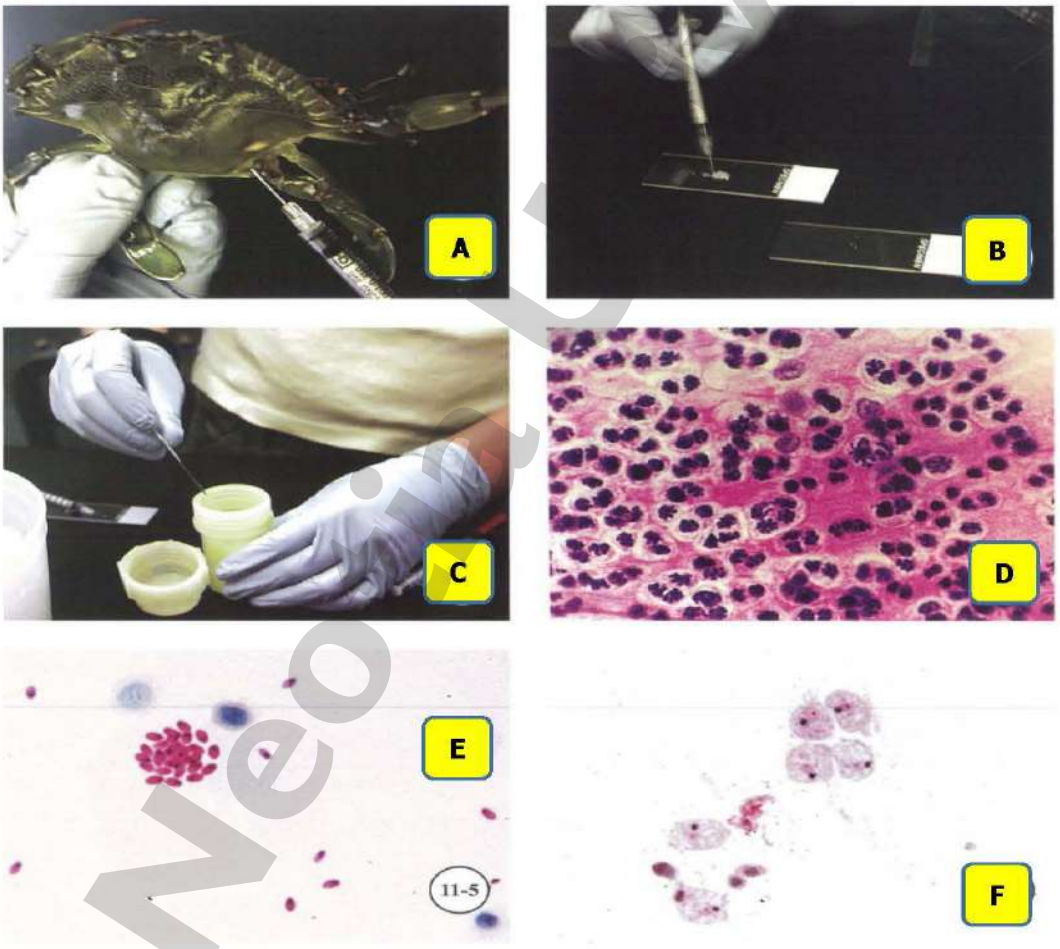
			
A juvenile *P. monodon* with distinctive white spots of WSD.	Carapace from a juvenile *P. monodon* with WSD. Calcareous deposits on the underside of the shell account for the white spots.	Histological section from the stomach of a juvenile *P.chinensis* infected with WSD. Prominent intranuclear inclusion bodies are abundant in the cuticular epithelium and subcuticular connective tissue of the organ (arrows)	Section of the gills from a juvenile *P. chinensis* with WSBV. Infected cells show developing and fully developed intranuclear inclusion bodies of WSBV (arrows).
			
A small juvenile *Penaeus stylirostris* showing gross signs of acute IHHN disease. Visible through the cuticle, especially on the abdomen, are multifocal white to buff colored lesions in the cuticular epithelium or subcutis (arrows). While such lesions are common in *P. stylirostris* with acute terminal IHHN disease, they are not pathognomonic for IHHN disease.	A low magnification photomicrograph (LM) of an H&E stained section of a juvenile *P. stylirostris* with severe acute IHHN disease. This section is through the cuticular epithelium and subcuticular connective tissues just dorsal and posterior to the heart. Numerous necrotic cells with pyknotic nuclei or with pathognomonic eosinophilic intranuclear inclusion bodies (Cowdry type A) are present (arrows).	Dorsal view of juvenile *P. vannamei* (preserved in Davidson's AFA) showing gross signs of IHHNV-caused RDS. Cuticular abnormalities of the sixth abdominal segment and tail fan are illustrated.	A high magnification of gills showing eosinophilic intranuclear inclusions (Cowdry type A inclusions or CAIs) that are pathognomonic for IHHNV infections.

			
Section of the hepatopancreas of *P. plebejus* displaying several hepatopancreas cells containing BMN-type intranuclear inclusion bodies.	High magnification of hepatopancreas from a PL of *P. monodon* with a severe infection by a BMN-type baculovirus. Most of the hepatopancreas cells display infected nuclei.	MBV occlusion bodies which appear as esosinophilic, generally multiple, spherical inclusion bodies in enormously hypertrophied nuclei (arrows).	Sections of the hepatopancreas of a PL of *P. japonicus* with severe BMN. Hepatopancreas tubules are mostly destroyed and the remaining tubule epithelial cells contain markedly hypertrophied nuclei that contain a single eosinophilic to pale basophilic, irregularly shaped inclusion body that fills the nucleus. BMNV infected nuclei also display diminished nuclear chromatin, marginated chromatin and absence of occlusion bodies that characterize infections by the occluded baculoviruses.

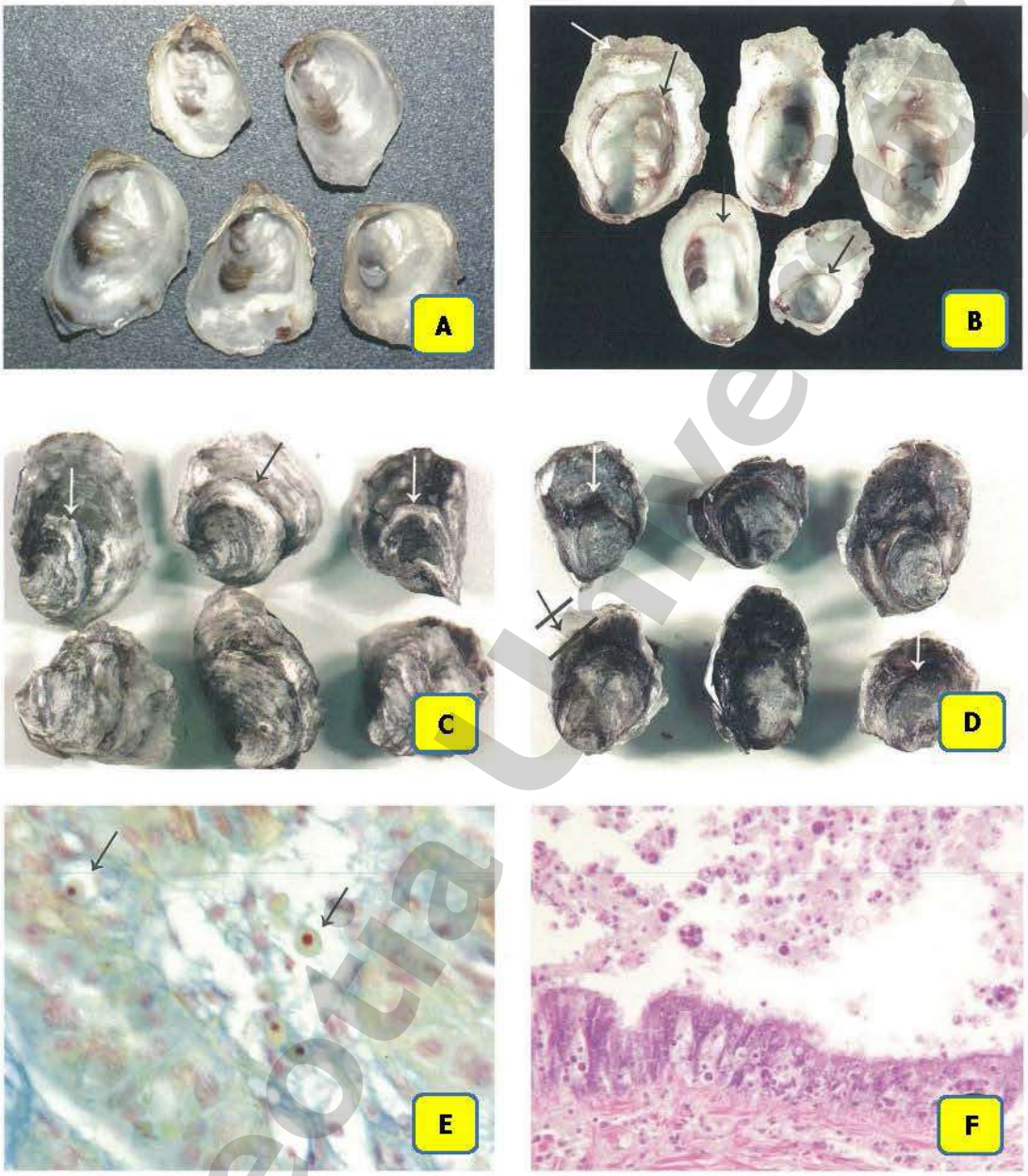
Black gill disease *L. vannamei* gills. A: Normal gill of L. vannamei; B: Black gill of diseased L. vannamei.



Normal gill lamella Black gill lamella.



A: Withdraw hemolymph from crab. B: Dispense and spread hemolymph onto poly-L-lysine coated slide. C: Place slides in fixative. D: Example of *Hematodinium* in crab hemolymph stained with Mayer's hematoxylin and eosin (MHE). E: Microsporidan in hemolymph stained with Ziehl Neelsen and methylene blue. F: *Paramoeba perniciosa* in hemolymph. stained with MHE.



A: Shells of normal juvenile oysters. B: Shells of JOD-infected oysters displaying conchiolinous shell lesions (black arrows); mantle recession (fouling of inside posterior margin of bottom shell) can be seen on shells of several JOD-infected oysters (white arrow). C: Shell checks on bottom valve (arrows). D: Shell checks on top valve (white arrows) and mantle recession (area between bars, black arrow). E: Mantle lesion with small round bodies (arrows) indicative of JOD-infected oyster stained with FPM. F: Mantle lesions. stained with MHE.

Video link: https://youtu.be/64LObclGnLY

Chapter 13
Disease Diagnosis of Finfish Based on Pathological Signs

Clinical Signs of Fish	Probable Causes
Skin:	
Red areas on the body surface	External parasites (*Ichthyophthirius/ Trichodina/ Oodinium/ Dactylogyrus*)
	Internal infection, abdominal dropsy
	Environmental damages (acid disease, caustic effect of chemicals etc)
	Due to handling
Excess mucous production	Ectoparasitic infection, environmental damages/ chemicals
Ulcerations	Handling methods (eg: netting), ectoparasites, bacteria
Cotton wool like white grey coating which collapse in air	Fungus (eg: *Saprolegnia*)
Velvet- like bluish white turbidity	Infestation with parasites (*Costia, Trichodina, Chlidonella, Gyrodactylus*, etc) Effect of chemical (Alkali)
Pearl to raspberry like growths	Lymphocystis
Long to short oval nodules originating deeper down, grey–white to whitish, black spots	Metacercarial cysts
Black colouration of whole sections of the body, usually starting posteriorly	Whirling disease, Viral hemorrhagic septicemia of trout

Blisters	Abdominal dropsy of cyprinids, bacterial kidney disease, furunculosis, ulcer disease
Lepidorthosis (Raised Scales)	Inflammation and swelling of the skin due to diseases caused by bacteria
Redness and prolapse of the anus, often exuding yellowish mucous when squeezed	Inflammation of intestine caused by viral or bacterial Infection Environmental irritants, unsuitable food irritating intestine, intestinal parasites (Eg: *Eimeria*), Furunculosis
Sunken eyes	KHV, severe internal illness, metabolic disturbances, starvation, chlorine poisoning
Opaqueness of cornea or eye	Infection due to *Streptococcus* sp., parasites (Metacercariae) or chemicals
Raised operculum	Tumour of thyroid gland or gill swelling
Frayed fins	Bacterial infection, metabolic disturbances, obstructed circulation due to internal infection
Discolouration in line or patch form	Muscular damage due to parasites (Micro/ Myxosporidia), Fungi (*Aphanomyces*) etc
Exophthalmos	Swelling behind the eye ball caused by infectious diseases, parasites or rarely of genetic origin (telescope fish)
Gills:	
Pallor	Anaemia, oxygen deficiency
Dark colouration of abnormal intensity	Circulatory obstruction due to pathogens or chemicals
Copious mucus secretion	Chemical irritant in water or due to parasites
Gill extremities destroyed, frayed, cauterised	Chemical irritants in the water or more rarely due to parasites

Marked swelling of individual gill filaments	Bacteria, avitaminosis, irritation by parasites (*Ichthyophthirius, Dactylogyrus*)
Yellowish nodules of pinhead size	Myxosporean cysts
Small white round spots	*Ichthyophthirius*, *Cryptocaryon*
Longish white spots	Gill crustaceans (*Ergasilus*) or *Dermocystidium* cysts
Cotton wool like coating on the gills, collapses in air	*Saprolegnia*
Brownish colouration, often at gill extremities	Gill Rot (*Branchiomyces*)
Whitish turbidity	*Costia*, *Chilodonella*, *Trichodina*
Bluish white, velvety coating of gills	*Oodinium*
Small worms, colourless to yellowish	Monogenetic Trematodes
Nodules	Tumour
Bubbles	Supersaturation of water
Gall Bladder:	
Presence of very motile, microscopically small unicellular organisms	*Hexamita* (Protozoan)
Presence of non-motile spores	Myxozoa, Microsporidia
Liver:	
Pale to yellowish discolouration	Viral hemorrhagic septicaemia (VHS), Lipoidosis, Infectious dropsy, Furunculosis, Bacterial Kidney Disease (BKD), Infectious Pancreatic Necrosis (IPN)
Patchy inflammation of liver, brownish spots	VHS, Necrosis, Cirrhosis, Furunculosis
Pin head to Pea-sized ball like cysts with worms	Cysts with plerocercoids of *Triaenophorus*, Nematode cysts
Yellowish white cysts, slightly large, without worms	Hepatoma
Whitish, minute cysts	*Ichthyosporidium*, Piscine tuberculosis

Yellowish, minute ball like cysts	Metacercaria
Round, long worms	Nematodes
Gut:	
Redness and inflammation of alimentary canal	Infections, parasites, poisoning, faulty feeding
Distension of stomach or parts of intestine	Disturbances of digestion, inflammation of intestine
Small yellowish nodules on the outer surface of the intestine	Anterior parts of acanthocephalans pushed through the intestinal walls
Swelling of pyloric caeca	Parasites, (usually cestodes)
Excessive mucus secretion of intestine	Intestinal irritation
Small, yellowish white nodules on interior surface of intestine	*Eimeria*, Myxosporeans
Round worms, white or yellow, adhering	Acanthocephalan (Thorny headed worm)
Long, white flat worms, segmented	Cestodes
Whitish, flat, unsegmented worms	Usually plerocercoid larvae of cestodes
Pale white, yellowish, reddish or brownish worms, flat, unsegmented, usually very small	Trematodes (flukes)
Small unicellular parasites in intestinal contents	*Hexamita* (Protozoa)
Eggs within intestinal contents	Usually eggs of worms
Kidney:	
Bloody inflammation of kidney, swelling	Viral Hemorrhagic Septicemia of Trout, Bacterial Kidney Disease
Cyst like swelling of kidney	Renal cysts
Kidney containing encysted helminth eggs	*Sanguinicola* (blood fluke)
White nodules in kidney	Visceral granuloma, *Ichthyosporidium*, Bacterial Kidney Disease
Head kidney abnormally thick, with grey colouration	*Sphaerospora*

Granulomas in kidney	Bacterial kidney disease
Loose, crunchy, white coloured deposits of Ca_3PO_4 resulting in the dilation of tubules/ ducts and thickening of glomerulus (Nephrocalcinosis)	CO_2 in water more than 12 ppm
Necrosis and mats of fungus in the initial stages developing into granulomas with few hyphae. Presence of giant cells fusion of macrophages in kidney	*Exophila* infection
Pancreas:	
Pancreas necrotic, muscle degeneration	IPN
Necrosis and haemorrhages	IHN
Lipid tissue associated with exocrine part (acinar cells)	Vitamin E deficiency or rancidity of feed
Spleen:	
Notably enlarged	Presence or consequence of an infection
Small white nodules on spleen	*Ichythosporidium*, Bacterial Kidney Disease, Tuberculosis
Gonads:	
Inflammation of gonads	Furunculosis, VHS, *Ichthyosporidium*, Abdominal dropsy of cyprinids, Poisoning
Cyst in ovary, necrosis/ fibrosis leading to sterility	*Pleistophora ovariae*
Round, coiled worms in between parts of gonads	Nematodes
Swim bladder:	
Loss of balance due to loss of control of gaseous secretion	Unknown, nutritional factors or excess dusty feed suspected
Chronic inflammation with necrosis and excess exudates in gas bladder	Unknown, Virus suspected
Oedema of walls of gas bladder	Chronic bacterial infections or other systemic infection

Parasites (Nematodes) and Cyst (*Eimeria*) in the lumen	
Heart:	
Yellowish- white ball like nodules in the heart	Metacercariae, Myxosporeans *Ichthyosporidium* (fungi), Tuberculosis
Worm eggs in heart blood	*Sanguinicola*
Lesions in heart muscle and pericarditis	Bacterial (BKD)
Cardiac oedema	Osmoregulatory failure Direct cardiac failure with renal Branchial circulatory failure
Haemorrhages	Virus
Resting spores and fungal hyphae in cardiac muscle resulting in cardiac failure	*Ichthyophonus*
Muscles:	
Muscles containing microscopic ball like cyst with spores	Microsporidia
Cyst formation, very often pigmented and mild fibrosis	Metacercaria
Muscles containing flat, unsegmented whitish worms	Cestode larvae
Parasites with major portion of head buried inside muscle	Anchor worm
Resting spores and fungal hyphae, chronic granuloma formation	*Ichthyohonus*
Liquefactive necrosis	Muscle parasites like microsporidians/ Myxosporeans
Ulcerations in muscle	Infectious abdominal dropsy, furunculosis
Small, white to grey-brown nodules	*Ichthyosporidium* (fungus)

Video link: https://youtu.be/6jR0Jqkct1U

Chapter 14

Disease Diagnosis of Shellfish Based on Pathological Signs

Clinical Signs of Shrimp	Probable Causes
Body colour or markings:	
Reddening of the legs and body	Gill associated virus (GAV) related disease, Vibriosis
Black marks or lesions	Healed wound, Bacterial shell disease Black splint disease
White spots in the cuticle	Non-viral conditions, Exotic viral disease, White Spot Syndrome Virus (WSSV)
White muscle	White cotton disease, Extreme pond temperatures (heat stress)
Red midgut	Haemocytic enteritis (gut infection)
Gill colour:	
Red gills	Stress
Black gills	Significant organic fouling in the pond bottom or algae die-off Blue-green algae growing on gill filaments Infectious damage to the filaments and melanisation Exposure to iron salts
Deformities or external problems:	
Tail cramping	High temperatures and/or salinities
Runts in the crop	Disease (haemocytic enteritis) or genetic growth variation
Tumours or abnormal growth(s) on the body	Genetic disease, chemical pollution
Behaviour:	
Abnormal swimming, burrowing, congregating	Temperatures (too cool or too hot), heat stress, low dissolved oxygen, sudden drop in pH, high salinity, thick algal bloom, shell or gill fouling
Prawns fail to moult	
Empty gut:	
Anorexia	Stress, rancid or poor-quality feed, lack of feed, poor water quality

HEALTH ASSESSMENT OF AQUATIC ANIMAL

Physiological Test:

Name of test	Activity	Sign of healthy seed
Feeding test	Place with preferred food	Should have full guts in 10–15 minutes
Swirl test	Swirled in water	Should realign and start swimming into the current
Salinity stress test	Expose to a salinity variation of 5-10 ppt for 2 hours	Should survive and resume feeding within 24 hours of the test
Temperature stress test	Keep with a temperature variation of 6-8^{0}c for 5-10 minutes	Should survive and recover quickly when put back in ambient water temperature.
Formalin stress test	Place in water having 100 ppm formalin with aeration	Should survive more than 80%
Size test	Measure the size	Should have uniform size

Note: Moulted one is less physiologically tolerant and is more susceptible to stress. Hence, assessments of larval quality can never be standardised during moult stage of shrimp.

Visual Observation of PL:

Criteria	Healthy seed	Unhealthy seed
Colour	Light grey/ black/ brown or transparent	Reddish/ pinkish blue reflects stressed post larvae.
Activity	Swim actively, does not clump together and remain evenly distributed. In a basin they may not move always but jump when mildly tapped on the container. When water current is created, the larvae try to move against the current.	They tend to settle in clumps. React feebly to gentle tap on container.
Feeding	Readily accepts and eat feed.	Reluctant to accept feed.

Microscopic Observation of PL:

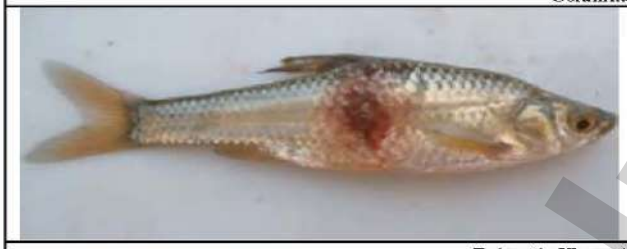
Criteria	Healthy seed	Unhealthy seed
Shell	Clean shell is a sign of regular moulting frequency and good health	Dirty shell or necrosis (wounds) on the shell or presence of protozoan.
Muscle	Clear, smooth and thick muscle completely fill the space between gut and the underside	Grainy and thin whitish or opaque muscle not filling the space below gut
Gut	Full gut with food	Empty or incompletely filled gut
Muscle to gut ratio	Tail muscle to hind gut ratio 4:1 or more	Ratio less than 4:1
Pigmentation	Chromatophores well defined and located along the mid ventral line	Chromatophores diffused or expanded
Appendages	Intact, without any deformity or having bent setae uropods remaining spread like a fan.	Broken or deformed appendages.

Video link: https://youtu.be/iypzpMSblaQ
https://youtu.be/DjrCY718g-w

Chapter 15
Finfish Disease Gallery

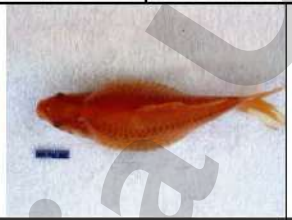


Columnaris disease




Epizootic Ulcerative Syndrome (EUS)

Dropsy



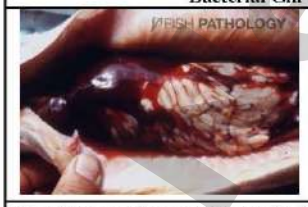
Motile Aeromonas Septicemia (MAS)

Myxoboliasis

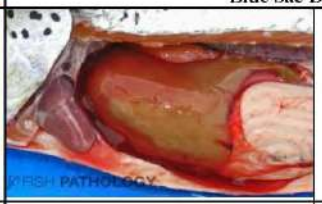
Argulas infestation


Bacterial Gill Disease (BGD)


Blue Sac Disease (BSD)



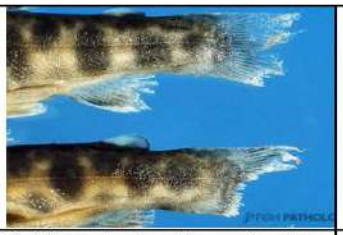
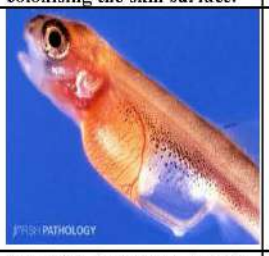
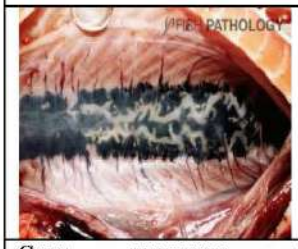
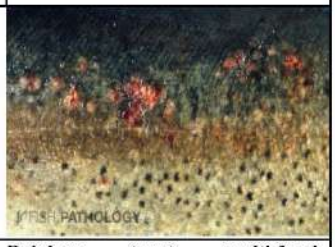
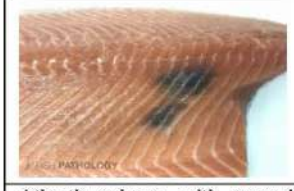

Cardiomyopathy syndrome in large Atlantic salmon. The sinus venosus in this fish has ruptured, leading to a blood-filled peritoneal cavity.



Heart and skeletal muscle inflammation (HSMI)

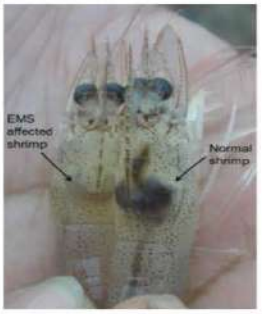
Atlantic salmon with cataract


Atlantic salmon (adult-1500 g) with BKD. Note the multiple granulomas in kidney. These severe lesions lead to renal failure.

			
Atlantic salmon with BKD. Note the multiple granulomas in spleen.	Rainbow trout with early stage infection with *F. psychrophilum*. Note the erosion in the caudal fin and peduncle.	*F. psychrophilum* infection in older rainbow trout. Note the single severe necrotic lesions in the muscle. Each raised fried egg-like lesion has a yellow-pigmented centre, a result of the large numbers of these yellow-pigmented bacteria (YPB) colonising the skin surface.	Gastric Dilation & Air Sacculitis Or "Bloat"
			
Rainbow trout with several gas bubbles in the anterior chamber. Note the haemorrhage both dorsally and ventrally, the latter "coning" through the ventral iris pore.	Unilateral exophthalmos in aquarium fish due to GBD.	Fry with large gas bubble behind the yolk sac. This animal was having trouble with its buoyancy.	Atlantic salmon smolt with HSS showing petechial haemorrhaging in the liver. Note also the pallor in the gills, a consequence of the haemorrhage.
			
Gross appearance of nephrocalcinosis. The kidney is swollen and grey with an irregular surface and white mineral deposits in the ureters.	Proliferative Gill Disease (PGD)in Atlantic salmon showing white spots (clubbing), distal third of filaments.	Rainbow trout with PKD. There is a marked hyperplasia of interstitium with the kidney thrown into bulbous ridges. The reddening is the result of secondary Yersinia infection.	Rainbow trout, multi-focal granulomatous dermatitis (Spawning Rash).
			
Atlantic salmon, with muscular melanosis.	Atlantic salmon, with peduncular melanosis.	Severe opercular erosion in farmed rainbow trout.	Atlantic salmon, juvenile with short operculum. Note the gill exposure.
			
Spinal fracture due to electric shock	Acute haemorrhagic dermatitis in rainbow trout, typical of what is called "summer strawberry disease".	Tenacibaculosis in fish	

Video link: https://youtu.be/RPWNDHYDhzU
https://youtu.be/QXIAc_v7WM4

Chapter 16
Shellfish Disease Gallery

			
White spot virus infected shrimp	Black gill disease (Fusarium disease)	Hepatopancreatic microsporidiasis	Bent/cramped tails or body cramp
			
Taura syndrome	Infectious myonecrosis	White tail disease	Black spot disease
			
Yellow head virus in shrimp	Vibriosis in shrimp	Mycobacterial infection	Acute hepatopancreatic necrosis / Early mortality syndrome (EMS)

Video link: https://youtu.be/--IPbIOvrNQ

Chapter 17
References

- Augustine, A., Benson, K., Pillai, D., Hameed, F. S., Sharma, K., Manoj, C. K., ... & Thulasikumari, S. (2020). Standard Operating Procedures for Aquatic Animal Health Management.
- Blazer, V. S., Fournie, J. W., Wolf, J. C., & Wolfe, M. J. (2007). Manual for the microscopic diagnosis of proliferative liver and skin lesions in the brown bullhead (Ameiurus nebulosus). *Pennsylvania Sea Grant, Pa.(ERL, GB 1291). Available from Pennsylvania Sea Grant at: http://seagrant. psu. edu/publications/technicaldocs/HistoFieldManual. pdf.*
- Blazer, V. S., Fournie, J. W., Wolf, J. C., & Wolfe, M. J. (2007). Manual for the microscopic diagnosis of proliferative liver and skin lesions in the brown bullhead (Ameiurus nebulosus). *Pennsylvania Sea Grant, Pa.(ERL, GB 1291). Available from Pennsylvania Sea Grant at: http://seagrant. psu. edu/publications/technicaldocs/HistoFieldManual. pdf.*
- Heil, N. P. (2004). Sample collection and submission. In *United States Fisheries and Wildlife Service Handbook of Aquatic Animal Health Procedures and Protocols Volume 2: National Wildlife Fish Health Survey Laboratory Procedures Manual.*
- Howard, D. W. (2004). *Histological techniques for marine bivalve mollusks and crustaceans* (Vol. 5). NOAA, National Ocean Service, National Centers for Coastal Ocean Service, Center for Coastal Environmental Health and Biomolecular Research, Cooperative Oxford Laboratory.
- Johnson, S. K. (1989). Handbook of shrimp diseases.
- Meyers, T. R. (Ed.). (2000). *Fish pathology section laboratory manual.* Alaska Department of Fish and Game, Commercial Fisheries Division.
- Mumford, S. L. (2004). Histology of finfish. *USFWS, Olympia Fish Health Center. Olympia. Washington.*
- Mumford, S., Heidel, J., Smith, C., Morrison, J., MacConnell, B., Blazer, B. (2007). Fish Histology and Histopathology.*USFWS-NCTC*
- OIE, (2013). Aquatic Animal Health Code.16th edn., World Organisation for Animal Health, Paris, France, pp. 284, ISBN 978-92-9044-916-4.
- Sarker, S. (2015). Bacteriology and histopathology of columnaris-like disease in cultured fish of West Bengal, India. Master's thesis, West Bengal University of Animal and Fishery Sciences, Kolkata.