

The Elasmobranch Husbandry Manual: Captive Care of Sharks, Rays and their Relatives

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Chapter 27

Histological and Histopathological Examination of Elasmobranchs: Emphasis on the Collection and Preparation of Tissues

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Abstract: An essential component of the husbandry and management of any captive population, including elasmobranchs, is an active program of disease surveillance and prevention. The foundation of the latter activity is the proper diagnosis of disease conditions including a postmortem (necropsy) examination of affected animals, incorporating the histological examination of a complete set of tissues. The necropsy examination should ideally be performed immediately following death or euthanasia, to prevent rapid autolysis due to a prolonged postmortem interval. Alternatively, the carcass may be refrigerated at temperatures of 4°C if the necropsy cannot be performed immediately following death of the animal, although a refrigerated carcass should be examined as soon as possible. Freezing is discouraged as a method of preservation of the carcass. A complete set of tissues should be collected for histological examination prior to a detailed gross examination of the tissues to prevent excessive handling that can result in artifactual changes of the tissue. Likewise, the tissues should be handled with care to avoid artifactual changes and dissected using forceps and a razor blade, scalpel blade, or sharp scissors prior to immediate placement in tissue fixative. A maximum thickness of 1.0 cm or a volume of 1.0 cm³ is preferred for the best fixation. A final volume of one part tissue to a minimum of 10 parts fixative (1:10) is highly recommended for the best results. A solution of 10% neutral-buffered formalin is the standard fixative for histopathological examination of animal tissues and may be used for the routine examination of elasmobranch tissues. However, acid-fixatives, such as Bouin's and Davidson's solutions, result in superior tissue fixation and cytological detail, and are often the preferred fixatives for elasmobranch tissues or piscine tissues in general. The histological examination of biopsy tissue, especially biopsies of the integument and gills, often provides valuable information and is recommended as a diagnostic procedure in elasmobranchs. The collection of tissues for electron-microscopic examination is not a routine procedure, although electron-microscopic examination is often required for a definitive or more complete pathological diagnosis. The most common fixatives for electron-microscopic examination are a 2% glutaraldehyde solution in a 0.1 M phosphate or cacodylate buffer, Trump's 4F:1G solution in a 0.1 M phosphate buffer, and 10% neutral-buffered formalin. The fixative of choice is Trump's 4F:1G solution, since it provides excellent fixation and the tissues can be held in the fixative for extended periods at 4°C. Similar to tissues collected for histological examination, a general guideline is fixation of one part tissue in a minimum of 10 parts fixative for a maximum of 1-2 hours at room temperature. In contrast to tissues collected for histological examination, tissues for electron-microscopic examination should not exceed a volume of 1.0 mm³. Confidence and accuracy of the pathological diagnosis not only depends on the proper collection and preparation of tissues, but is also dependent on the expertise of the pathologist. In this context, the pathologist should be selected based on a familiarity and expertise with the normal anatomy and physiology, husbandry, medicine, and pathology/diseases of elasmobranchs.

Although the surveillance and prevention of disease are essential components of the husbandry and management of captive populations, these activities are often absent or otherwise not properly performed or applied to aquatic animal populations, including elasmobranchs. The foundation of any disease surveillance and prevention program is the proper diagnosis of disease conditions, including a complete evaluation of the history and husbandry of the captive population, analysis of the water quality parameters, and an antemortem physical examination or a complete postmortem (necropsy) examination of the affected animals. Components of the antemortem and postmortem examination include a detailed gross examination; cytological examination of tissue scrapings and/or imprints of the external (gills and integument) and internal tissues; and the collection of tissues for hematological, chemical, toxicological, microbiological (viral, bacterial, parasitic, and mycotic), and histological evaluation.

In the event of mortality or euthanasia, a complete necropsy examination is always recommended, since the failure to perform a complete necropsy often results in an incorrect diagnosis of the primary disease condition or the cause of the disease. An example of an improper postmortem diagnostic effort is the evaluation of cutaneous and branchial wet-mount preparations without the benefit of a complete necropsy examination or the histological evaluation of a limited number of tissues. This limited examination may reveal an external parasitic infection that may not be the primary cause of the disease, since the majority of infectious disease conditions in captive populations are secondary to poor husbandry, stress, or other primary disease conditions. The consequences of an incorrect diagnosis are obvious and may result in an avoidable loss of animals due to additional morbidity and mortality in the population. Likewise, treatment of the secondary infectious disease will generally not prevent recurrence of the primary disease condition. Therefore, this discussion will focus on the proper collection and preparation of tissue samples for histological examination, increasing the accuracy, and consequently the confidence, of the pathological diagnosis.

BASIC CONCEPTS OF DISEASE

Prior to any discussion of pathology and disease in an individual organism or population, a conceptual framework of disease needs to be established by definition of the pertinent terminology. Therefore, disease can be defined as any definitive morbid

condition or process that has a characteristic set of symptoms or qualities, whereas pathology is the study of disease. The various aspects of pathology include the cause or etiology, the developmental process or pathogenesis, the biochemical and morphological alterations or lesions of the cells and tissues, and the functional significance or clinical consequence of these alterations. The etiology of disease may be intrinsic (genetic) or extrinsic (acquired); the latter includes diseases due to infectious, environmental, toxic, physical or traumatic, metabolic, and/or nutritional etiologies.

Neoplastic disease may have an extrinsic and/or intrinsic component, whereas a disease with an uncertain or unknown etiology is referred to as an idiopathic disease. The cause of any particular disease may be due to a single etiology, such as a highly virulent infectious agent, or multiple etiologies. For example, fish exposed to poor water quality or low concentrations of a toxin are generally more susceptible to a primary and/or secondary infectious disease. Disease can be further classified according to the progression and severity of the condition. Acute disease has a rapid onset and progression, whereas chronic disease has a slow progression and long duration. Disease that is neither acute nor chronic may be classified as subacute or subchronic, whereas disease that has an extremely rapid progression can be considered peracute. Clinical disease is grossly perceptible or conspicuously apparent and characterized by observations and/or the results of tests, whereas subclinical disease is not apparent or does not result in clinical manifestations and is difficult to characterize. Subclinical disease may progress to clinical disease.

Infection is often used synonymously with disease but is more correctly defined as the invasion and colonization of the tissues by microbial pathogens and the consequent response of the host to this event. A pathogen is any organism capable of causing disease, whereas pathogenicity is the ability of an organism to produce disease. Pathogenicity of an infectious agent is dependent on the contagious and invasive properties of the pathogen, and the ability of the pathogen to resist defense mechanisms of the host that will vary with a particular strain. Infection that results in apparent symptoms, i.e., disease, is often referred to as clinical infection but is more correctly characterized as a clinical disease due to an infectious etiology. In contrast, a subclinical infection is synonymous with asymptomatic infection that is generally not grossly perceptible or conspicuous and does not result in disease. Therefore, the detection or

presence of any infectious agent does not imply the presence of disease. Asymptomatic infections may progress to clinical infections or may remain subclinical, although the host may function as a reservoir of infection to other members of the population, referred to as the carrier state. Furthermore, exposure to infectious agents is a normal and continual event that does not necessarily result in infection or clinical disease during the lifespan of any individual organism. The manifestation of clinical disease in a population or epizootiology is dependent on a complex interaction between the host organism, the environment, and the pathogen. For example, the ability of a viral pathogen to cause disease is dependent on the status of the host, including species, age, and life stage; water quality parameters; and the pathogenicity of the viral strain.

Pathogens are normal components of the aquatic ecosystem that have coexisted and evolved with the host in the natural environment, and generally do not result in serious disease within the wild population. However, captivity often provides conditions that affect the complex interaction of the host and pathogen. These conditions often exacerbate the manifestation of disease in a captive population, but do not create the host-pathogen interaction.

There are several classifications of the clinical or pathological diagnosis. A definitive diagnosis of vibriosis would be an appropriate diagnosis in a shark with a primary disease condition due to a *Vibrio* sp. infection. In contrast, a morphological diagnosis only characterizes the lesions of the specific tissues that are affected by the disease, whereas an etiological diagnosis associates the morphological diagnosis with the causative or etiological agent of the lesions. For example, an ulcerative dermatitis would be a correct morphological diagnosis if the disease condition resulted in cutaneous inflammation with the development of cutaneous ulcers. If the cutaneous inflammation and subsequent ulceration were due to a *Vibrio* sp. infection, the correct etiological diagnosis would be a bacterial ulcerative dermatitis due to a *Vibrio* sp., although the definitive diagnosis for this disease condition would be a cutaneous vibriosis. The distinction is that the etiological agent characterizes or qualifies the morphological lesion in the etiological diagnosis, whereas the affected tissue or organ characterizes or identifies the location of the disease condition in the definitive diagnosis.

A morphological diagnosis can generally be obtained by the gross and histological examination of tissues if there are structural alterations to the tissues, but cannot be obtained if cellular lesions are limited to functional (i.e., subcellular or biochemical) alterations. The latter effect may occur with an acute environmental or toxic insult. In this context, the histological examination of tissues may not result in an etiological diagnosis or definitive diagnosis, since the latter diagnoses often require ancillary tests in addition to the histological examination.

The accuracy and confidence of any pathological diagnosis is dependent on the proper collection and preparation of tissue samples.

COLLECTION AND PREPARATION OF TISSUES

Those that are not familiar with the normal anatomy of elasmobranchs should consult the manuals by Ashley and Chiasson (1988), Gans and Parsons (1964), and Harrison (1949), prior to the necropsy examination. Hamlett (1999) has summarized the biology of elasmobranchs, including a review of the various organ systems.

Necropsy

A necropsy examination should ideally be performed immediately following death or euthanasia, to prevent rapid autolysis due to a prolonged postmortem interval. Autolysis will occur in all tissues but is especially rapid in the gills, gastrointestinal tract, brain, and spinal cord. For example, structural alterations of the gills can occur within five minutes of death (Ferguson, 1989). Alternatively, the carcass should be removed from the water without further delay and refrigerated at temperatures of 4°C if the necropsy cannot be performed immediately. However, a refrigerated carcass should be examined as soon as possible, since refrigeration of poikilotherms (i.e., cold-blooded animals) only decreases the rate of autolysis, rather than preventing it altogether. Animals that have been dead for several hours (e.g., overnight) should be necropsied without further delay. Freezing is discouraged as a method of carcass preservation since freezing disrupts the normal structural integrity of cells and tissues (freezing artifact), although a frozen carcass can often be used for microbiological and toxicological analyses.

Tissue collection

It is recommended that a complete set of tissues (i.e., representative tissues from all of the organ systems) be collected for histological examination, prior to a detailed gross examination of the tissues, to prevent excessive handling that can result in artifactual changes of the tissue. For example, a cursory examination of the gills should be followed by collection of gill tissue for histological examination, prior to further gross examination and wet-mount or cytological examination of the gills. Additional sections of any tissue may be taken or substituted for the original sample(s), as necessary, during the gross examination. The complete set of tissues should always include any gross lesions and the adjacent normal tissue. For example, sections of a gross lesion from the liver, such as a focus of capsular and subcapsular hepatic hemorrhage and inflammation, should include the lesion, border of the lesion, and ~1.0 cm section of the grossly normal adjacent tissue. An additional section of normal liver that is not adjacent to the gross lesion should also be collected and submitted for histological examination. Care should be exercised during the necropsy examination to avoid contact of the tissues with water, saline solutions, other chemicals, or other tissues from the same animal or different animals, including gastrointestinal contents. The tissues should be handled with care to avoid artifactual changes and dissected using forceps and a razor blade, scalpel blade, or sharp scissors, prior to immediate placement in tissue fixative. Razor blades and scalpel blades should ideally be replaced after two or three uses to avoid artifactual changes. A maximum tissue thickness of 1.0 cm, or a volume of 1.0 cm³, is preferred for the best fixation.

The pathologist often prefers the identification of tissues prior to submission for histological examination. This procedure will prevent confusion concerning origination of the tissue, especially if a lesion obscures the normal microscopic anatomy of the tissue or if multiple sections of a single organ are procured for examination. Tissues can be placed in tissue cassettes or tissue bags labeled with pencil prior to fixation. Alternatively, separate vials or containers can be used for larger tissues such as the entire heart or brain. For juvenile fishes, the entire carcass can be immersed in tissue fixative following exposure of the internal viscera by a ventral midline incision. Containers should have a wide opening to facilitate placement of the tissues into the container without excess manipulation or handling. A final volume of one part tissue to a minimum of 10 parts fixative (1:10) is highly recommended for the best results. Tissues

preserved in fixative can be held at room temperature prior to shipment or submission to the laboratory.

Tissue fixation

The various fixatives and formulations that are used for the histological examination of tissues have previously been discussed in detail and should be consulted as necessary (Bancroft and Cook, 1994; Hopwood, 1990; Presnell and Schreiber, 1997). A solution of 10% neutral-buffered formalin is the standard fixative for histopathological examination of animal tissues and may be used for the routine examination of elasmobranch tissues, but is not always the preferred fixative. The advantages of formalin are related to convenience, since it is commercially available and is generally the fixative of choice for various histochemical stains. Tissues fixed in formalin can be held in the fixative, pending submission to the diagnostic laboratory, and can be used for electron-microscopic (ultrastructural) examination if necessary. However, formalin often results in less than ideal tissue fixation and cytological detail, sometimes compromising the accuracy of the histological examination and, therefore, the confidence of the pathological diagnosis.

In contrast to formalin, the acid-fixatives, such as Bouin's and Davidson's solutions, result in superior tissue fixation and cytological detail and are often the preferred fixatives for elasmobranch tissues and piscine tissues in general. More specifically, Bouin's solution is the preferred fixative for the histological examination of the eyes, brain and spinal cord, and whole juvenile fishes. Since Bouin's solution results in demineralization of tissues, it is the fixative of choice for mineralized tissues such as cartilage and integument. However, tissues fixed in acid-fixatives need to be placed in 70% ethanol after a maximum fixation of 12-48 hours to prevent over-fixation of the tissues. A maximum fixation of 24 hours is recommended for small sections of tissue. Acid-fixatives preclude the use of various histochemical stains and the electron-microscopic examination of tissues, due to the harsh nature of the fixatives. Bouin's solution contains picric acid, which is explosive when dry. Therefore, picric acid should be purchased as an aqueous solution and picric acid and Bouin's solution should be properly stored and discarded to prevent drying. Adjustments to the final pH and osmolarity of the fixative, to coincide with the pH and osmolarity of the extracellular fluid of the species to be examined, have often been recommended by histologists. However, this is not

necessary for routine diagnostic procedures, regardless of the fixative, since the cytological changes that may occur are minimal or insignificant and do not affect examination of the tissues.

Due to the advantages and disadvantages of the various fixatives, collection of a duplicate set of tissues in formalin and an acid-fixative (preferably Bouin's solution) is recommended, especially for large animals such as elasmobranchs. One or both sets of tissues may be submitted to the diagnostic laboratory with the understanding that only one set of tissues (preferably the tissues fixed in Bouin's solution) will be processed for histological examination, whereas the additional set will be saved for future examination or staining as necessary. Tissues may be placed in a smaller container with a small amount of fixative (such as formalin, for tissues initially fixed in formalin, or 70% ethanol, for tissues initially fixed in Bouin's solution), following adequate fixation for shipment to the diagnostic laboratory. The container should permit easy placement and removal of the tissues. Sealing the opening of the container and the lid with Parafilm (American National Can Company, Chicago, Illinois) and placing the container(s) in a well-sealed plastic bag will prevent leakage during shipment.

Tissue fixation for electron microscopy

The various fixatives and formulations used for electron-microscopic examination of tissues have previously been discussed in detail and should be consulted as necessary (Dykstra, 1993; Hayat, 2000; Hopwood and Milne, 1991). The collection of tissues for electron-microscopic examination is not a routine procedure, although electron-microscopic examination is often required for a definitive or more complete pathological diagnosis. Ideally, tissues that exhibit unusual gross lesions should be collected for electron-microscopic examination pending the results of the histological examination.

The most common fixatives for electron-microscopic examination are a 2% glutaraldehyde solution in a 0.1 M phosphate or cacodylate buffer, Trump's 4F:1G solution in a 0.1 M phosphate buffer, and 10% neutral-buffered formalin (Dykstra, 1993). The fixative of choice is Trump's 4F:1G solution, since it provides excellent fixation and the tissues can be held in the fixative for extended periods at 4°C (Dykstra, 1993). Phosphate buffers are generally preferred over cacodylate buffers, since the latter contain arsenic and are therefore toxic. Similar to tissues collected for histological examination, a general guideline is the fixation of one part tissue in

a minimum of 10 parts fixative for a maximum of 1-2 hours at room temperature. If glutaraldehyde was used as the fixative, tissues should then be placed in a 0.1 M buffer solution (using the same buffer that was used for fixation) for washing or storage prior to submission. The tissues should be collected using a sharp razor blade to avoid artifactual changes. In contrast to tissues collected for histological examination, tissues for electron-microscopic examination should not exceed a volume of 1.0 mm³. It should be emphasized that electron-microscopic examination is more expensive and less rapid than a routine histological examination.

Biopsy

A tissue sample taken from a living animal is referred to as a biopsy. The histological examination of biopsy samples, especially biopsies of the integument and gills, can provide valuable information and is therefore recommended as a diagnostic procedure in elasmobranchs. As the term implies, an excisional biopsy is simply the procurement of tissue by excision, using appropriate surgical instruments such as a scalpel blade. The collection of a small, circular section of integument is referred to as a punch biopsy. The punch biopsy is performed using a tissue punch, which is a specialized instrument for cutting and removing a section of the integument. Finally, the collection of a tissue core using a needle is referred to as a needle biopsy. Needle biopsies are generally used for the collection of tissue from the internal viscera, but are not the preferred procedure for the collection of integument and gill samples.

Cutaneous neoplasms are often raised lesions that can simply be excised at the base of the lesion. Cutaneous biopsy sites can be closed with an appropriate absorbable suture material, although this is generally not necessary and is often difficult due to the relative lack of cutaneous elasticity, especially of the dorso-lateral integument. The presence of dermal scales further complicates this procedure. Application of a topical antibiotic ointment to the biopsy site and/or a prophylactic injection of antibiotic can often follow a biopsy procedure, but is generally not necessary nor indicated. However, the systemic administration of antibiotics to elasmobranchs with ulcerative lesions, due to a primary or secondary bacterial etiology, is recommended.

The procurement of cutaneous scrapings is considered a biopsy procedure. Scrapings may be collected and placed in tissue fixative for histological

examination. Finally, gill biopsies are simple to perform and provide highly diagnostic information. The collection of branchial tissue should be limited to excision of the proximal aspects (or tips) of the primary filaments, using sharp scissors.

The collection of needle or wedge biopsies, from the internal viscera, is more difficult and generally provides less diagnostic information than cutaneous or gill biopsies, but can be performed if other diagnostic techniques (e.g., an evaluation of the history, water quality, cutaneous or gill biopsies, wet-mount preparations, etc.) do not provide adequate diagnostic information. The collection of internal biopsies is dependent on a knowledge of elasmobranch anatomy and/or the use of imaging techniques such as radiography or ultrasonography to locate the organ(s). However, the collection of biopsy samples using endoscopy, to evaluate the internal organs, is generally more successful and provides the best results.

The collection and preparation of biopsy samples is similar to the collection of tissues during the necropsy examination, although the small amount of tissue that is obtained generally precludes the collection of a duplicate set of tissues. Therefore, one fixative should be selected and formalin is generally the preferred fixative. Biopsy procedures that result in the procurement of small tissue samples, such as samples obtained using a needle biopsy, endoscopy, or cutaneous scraping, should be placed in a labeled tissue cassette to prevent loss of samples.

SELECTION OF THE PATHOLOGIST

Confidence and accuracy of the pathological diagnosis not only depend on the proper collection and preparation of tissues, but are also dependent on the expertise of the pathologist. In this context, the pathologist should be selected based on a familiarity and expertise with the normal anatomy and physiology, husbandry, medicine, and pathology of elasmobranchs. Additional factors to evaluate during the selection of a diagnostic laboratory or pathologist include the availability of the pathologist, enthusiasm of the pathologist to work closely with the client, quality of the report, time required for reporting the results, cost of the examination, and ability to recommend and perform additional diagnostic procedures as necessary. In general, quality should not be sacrificed for convenience.

The pathologist should be considered a member of the husbandry team and a client-pathologist relationship should be developed and fostered for the best results. Both the pathologist and the client should be readily available to discuss the results of the pathological examination.

A detailed pathological report, describing the morphological lesions and the clinical significance of those lesions, is not an unreasonable request. The report should include recommendations on management and/or treatment strategies, if possible, or the need for additional diagnostic procedures. However, it must be understood that the histological examination of tissues may not result in a definitive diagnosis or even a morphological diagnosis in all cases requiring additional diagnostic procedures or ancillary tests.

The time required for completion of the pathological report should be discussed with the pathologist, although requests by the client should be reasonable for routine cases. In contrast, a more rapid report is not an unreasonable request in the event of a progressive severe morbidity and/or mortality in the population. A reasonable cost for the examination is dependent on the various diagnostic procedures that are requested and performed, but should be similar to the cost of the same procedures in other veterinary species.

The pathologist or diagnostic laboratory should have the capability to perform additional diagnostic procedures including additional histochemical staining; electron-microscopic examination of tissues; and the hematological, chemical (clinical chemistry), toxicological, and microbiological analyses of samples.

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