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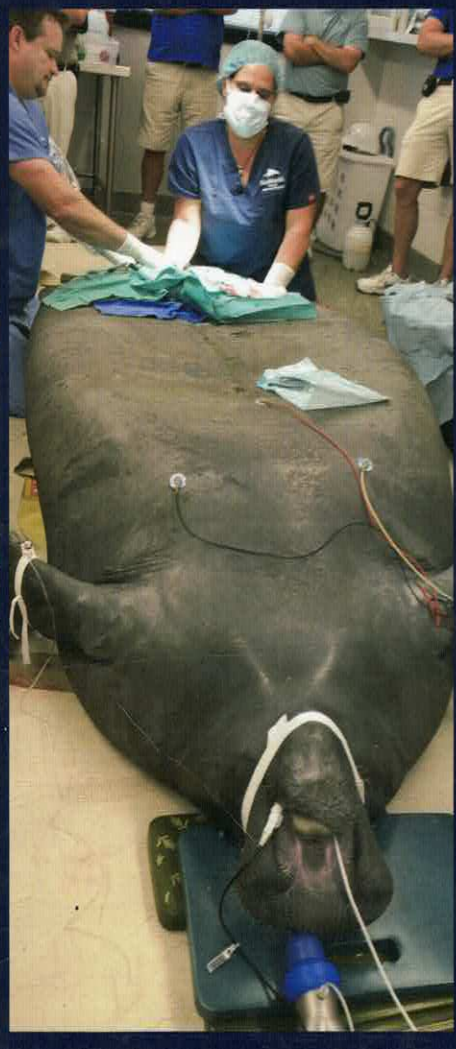


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MARINE MAMMAL GROSS NECROPSY

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Introduction

The primary goal of a necropsy is to establish cause of death. Yet, of equal importance is the opportunity to collect both samples (to archive) and data (to catalog) on subclinical diseases and life history parameters, including reproductive status, age, diet and nutritional state, anatomy, and genetics. These data can be used to establish disease patterns, document the effects of human interactions, and identify endemic and novel diseases in marine mammals. Review of necropsy findings and results from ancillary diagnostic studies can ultimately direct management strategies to mitigate factors causing disease or death. Over the last decade, there has been rapid development, refinement, and application of novel diagnostic and research modalities that have significantly advanced the recognition of specific disorders in dead marine mammals. In addition to conventional diagnostic studies of necropsy, histopathology, bacteriology, toxicology, virology, and molecular studies, incorporation of postmortem imaging investigations (by computed tomography [CT] or magnetic resonance imaging [MRI]; Moore et al. 2009; Dennison et al. 2012; see **Chapter 24**), mass spectrophotometry analysis of intravascular gas bubbles (Bernaldo de Quiros et al. 2013a and b), molecular sequencing, protocols to standardize carcass evaluation for evidence of anthropogenic interactions (Moore et al. 2013), and ultrastructural studies of ears to assess for auditory injury (Morell et al. 2015, 2017) are contributing invaluable information to marine mammal health and disease data.

The focus of this chapter is to provide an overview of gross necropsy and specimen collection protocols for marine mammals. It is not our intention here to develop new protocols, but to outline the more important aspects of a necropsy examination and reference some of the excellent and detailed protocols already published. It is important to note that collection and retention of salvaged marine mammals

and tissues may require appropriate permits, and prior to commencing any postmortem investigations, authorization should definitely be sought. In North America, for example, this work is under the auspices of the Marine Mammal Health and Stranding Response Program of the National Oceanic and Atmospheric Administration (USA), Department of Fisheries and Oceans (Canada), and Procuraduría Federal de Protección al Ambiente (Mexico). Most developed countries have similar national and/or regional agencies that should be consulted (see **Chapter 1 and Appendix 5**). International organizations such as the International Whaling Commission (IWC), International Council for Exploration of the Seas (ICES), and Arctic Monitoring and Assessment Program (AMAP) may provide further guidance. With the advent of specialized research and reference diagnostic laboratories throughout the world, consultation with the appropriate federal or national authority is strongly recommended prior to any international shipment, to ensure that authorizations and declarations are in place prior to transport. All cetaceans receive protections under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES; http://www.cites.org) and are designated a minimum status of CITES Appendix II, when appropriate export permits are warranted for transnational shipments. In addition, all CITES Appendix I designated animals require both CITES import and export permits listing the appropriate scientific name of the species and the nature of the tissue samples.

Necropsy Examinations and Specimen Collection

Logistics

The diversity of marine mammal species, oceanographic, climatic, and coastal features, and access to local stranding sites, can present unique challenges to recovery and postmortem examination of dead marine mammals. Animals may be reported offshore or beach cast in remote and occasionally inhospitable locations. Human safety in pursuing necropsy of these animals is paramount, and efforts to secure and tow animals to more accessible beach or boat ramp sites may be contingent on the postmortem state of the animal, financial resources, enforcement or forensic investigations, boat availability, and whether the animals are endangered, threatened, or of intrinsic scientific interest. In populated areas, there is a balance in controlling access around the necropsy site to ensure public safety, while still educating and increasing public awareness about the benefits of conducting postmortem examinations and threats to marine mammals. A number of infectious agents are recognized in marine mammals, which may pose significant public health concerns (see **Chapter 4**), so use of appropriate personal protective equipment (PPE) is recommended. Other potential risks include scavengers, such as bears in remote areas and sharks around floating carcasses. Ideally, carcasses should be recovered and transported to a diagnostic or research facility for necropsy, under controlled and secured conditions. Prior to movement, carcasses should be photographed where first discovered, as this may provide insight into events at death.

For some larger animals, field postmortem may be the only option available, with personnel and equipment transported to the carcass location. Field kits should be strategically deployed in caches along larger coastal regions; or a subset of instruments, sample containers, and necropsy data sheets prepared, at the ready, and transported at the time of necropsy. A list of equipment is provided in **Box 13.1**. Having prepacked necropsy kits allows rapid response to carcasses when field conditions are suitable (e.g., low or ebb tide, no precipitation). Since some stranding locations may be difficult to access, using a backpack, tool kit, or roller-pack to carry equipment is useful.

The extent of postmortem examination and tissue sampling of an animal may also be determined by the position of the animal on the beach (recumbency), carcass code (**Table 13.1**), availability of heavy equipment, and necropsy team experience. For larger animals, animals may be floated, repositioned, and secured by long line on a beach at high tide, with access for necropsy during ebb and low tides. Injection of compressed air into the abdomen can help refloat and move large carcasses where tidal range is limited. Smaller animals should be removed to above the high tide mark.

Smaller animals may be frozen intact for transport or necropsy at a later date. Logistically, this may facilitate transfer and ultimate postmortem examination of an animal; however, prolonged freezing or freeze–thaw may result in loss of more fastidious pathogens and compromise histopathology results. When freezing is necessary prior to transport of a carcass for complete necropsy, it may be possible to collect a subset of diagnostic samples before freezing, so that histopathology and valuable data on pathogens are not compromised by freeze–thaw degradation.

Prior to mass stranding events, contingency plans should be prepared, (practiced if possible), and at the ready, to ensure that prompt necropsies and targeted sampling are performed on any individuals that die. Prepare a plan for carcass selections as well, to ensure that in the event that a large number of animals die, resources and personnel are not overwhelmed (Jepson and Deaville 2017b). It is important to assess carcass code (**Table 13.1** details carcass code conditions) during these events to determine if mortalities are ongoing over time, or occurred simultaneously, and to determine the relative value of each necropsy examination. Mass strandings associated with catastrophic environmental or emergency events may quickly overwhelm local or regional resources, and activation of the Incident Command System (ICS) may also occur (see **Chapter 2**). Prior training and familiarity with this scheme should be encouraged for personnel involved with stranding and response programs.

At the time of a field necropsy for a large animal, teams may be identified and tasked with completing morphometric

BOX 13.1 NECROPSY AND SAMPLING EQUIPMENT CHECKLIST*

- ☐ Morphometric data sheets, gross necropsy forms, human interaction forms, and sample collection checklists
- ☐ Dissecting instruments, scalpel handles and blades, scissors, forceps, knives
- ☐ Sharpening steel and oil stones
- ☐ Flensing knives with retraction hooks, chain, reciprocating saw or hacksaw, hammers, chisels
- ☐ Retractors and come-alongs with lengths of rope (up to 20 m)
- ☐ Sterile instruments, propane torch/gas burner, and searing spatula for sterile culture collection
- ☐ Flood lamps, gas generator, flashlights, and/or headlamps with extra batteries and light bulbs
- ☐ 10% neutral buffered formalin (1–10 L)
- ☐ 4% buffered glutaraldehyde or suitable EM fixative (10–20 ml split in multiple small vials)
- ☐ 20% DMSO/saturated saline solution for genetic analysis (5 ml)
- ☐ Isopropyl alcohol for flaming instruments
- ☐ RNAlater (Thermo Fisher Scientific) for samples for future molecular analysis (5–20 ml split in multiple small vials)
- ☐ Sample collection containers with lids, including ice chest, dry ice, and liquid nitrogen (if possible)
- ☐ Bacterial and viral culture swabs with transport media
- ☐ Red top serum tubes for fluid, blood, and urine collection
- ☐ Aluminum foil, Teflon bags, and plastic bags/Whirl-Paks for freezing tissues
- ☐ Paper for notes, labels, and waterproof (Sharpie) marking pens
- ☐ Tape measure (metric), at least 20 m long, and small 12–15 cm plastic rulers
- ☐ Personal protective equipment (PPE): coveralls, aprons, boots, gloves, caps, masks, protective eye and head gear
- ☐ Digital camera, extra batteries, with additional memory cards
- ☐ Labels to identify digital images
- ☐ First aid kit
- ☐ Plastic tarps, 10 m in length
- ☐ Plastic tape and pylons to cordon off necropsy site
- ☐ Ice chest or cooler with ice to hold fresh samples
- ☐ Garbage bags, dish soap, disinfectant, scrub brushes, paper towels for cleanup

*Note: This equipment checklist represents an ideal situation. Postmortem exams can be completed with less equipment.

data sheets, external photo-documentation, laying out instruments, preparing disinfectant washes, identifying a knife sharpening area, managing sample disposition forms, labeling sampling containers and bags, and preparing a tissue dissection station. For field postmortems, the immediate area around the carcass should be assessed, and workstations and rest or refreshment areas identified. Instruments should be sorted and laid out in a readily accessible flat surface area with cleaning and sharpening stations nearby. A separate area identified for tissue subsampling, inventory, and disposition should be conveniently located to facilitate harvest, processing, and transfer of tissues to appropriate preservatives or placement in labeled plastic bags for freezing. Ideally, up to three individual replicate samples of each tissue should be archived for diagnostic studies, research investigations, and legacy collections. As instruments are used and replaced during the postmortem examination, it is imperative that they are appropriately cleaned and, in the case of knives, sharpened and ready for reuse during the necropsy. Smaller instruments may be rinsed, scrubbed, and then placed in a tray of disinfectant or laid out for reuse. Knives should never, even for a short time, be put down on a carcass.

Protocols, Data, and Forms

There is no single template or necropsy protocol that encompasses all marine mammals; however, species-specific necropsy protocols and tissue sampling procedures to screen for specific entities have been developed (**Table 13.2**). Necropsy protocols should be standardized as much as possible to facilitate cross-species and regional analyses of findings, data sharing, and research development.

Before any tissues are incised, a thorough external examination of the animal should be conducted to assess nutritional status, postmortem code, and record and photograph any gross lesions. It is helpful to assign a number to each external lesion. This allows linkage of photographs, samples, measurements, and descriptions as the case material is processed, analyzed, and reported. It is important to distinguish between lesions that were present at the time of death and

Table 13.1 Classification of Carcass Condition

Code	Definition	Gross Appearance	Specimen Collection	Interpretation
1	Live		Morphometrics, blood, biopsies, urine, infectious diseases, diagnostic imaging	
2	Freshly dead "edible"	No bloating; minimal drying and wrinkling of epidermis in cetaceans and manatees or dermis and epidermis in pinnipeds and otters, and of eyes and mucous membranes; muscles firm; blubber firm and white or yellow; internal organs intact; liver still with physical integrity	All types of specimens should be collected	Bacterial overgrowth may be observed on cultures or histology; some autolysis noted on histology
3	Moderate decomposition	Slight bloating with tongue and penis protruding; some skin sloughing and cracking; eyes sunken; blubber may be blood-tinged; muscles soft; all internal organs including liver still have gross integrity but are soft and friable	Morphometrics, gross pathology, parasitology, genetics, life history, some histology	Autolysis often masks histological assessment; decomposition may alter enzymatic, biochemical, and chemical analyses, including lipid quality and quantity
4	Advanced decomposition	Bloated; missing patches of epidermis and hair; internal organs show lack of integrity and are extremely friable; blubber with gas pockets and pooled oil	Morphometrics, gross pathology, parasitology, genetics, life history	Autolysis often masks cause of death; bloating and autolysis may alter morphometrics
5	Severe decomposition	Mummified; skeletal	Limited morphometrics, age, skeletal pathology, genetics	Cause of death only rarely determined

those that were "added" to the animal postmortem prior to the necropsy, such as those inflicted by tow ropes, lifting straps, and gravity within textured body bags.

Decomposition

Carcass code should be determined as a first step in a necropsy examination (**Table 13.1**). The carcass code or degree of postmortem decomposition will dictate the extent of field morphometrics, tissue sampling, and interpretation of diagnostic findings (Geraci and Lounsbury 2005). In large whales, postmortem gas may exert sufficient pressure to extrude the tongue, rectum, umbilicus, penis, vagina, or uterus, and caution should be exercised with gentle incision to allow for slow release of the gas.

The blubber layer may be attenuated and discolored through decomposition and liquefaction, hindering quantitative and qualitative assessment of the nutritional status of the animal. In those animals that present in code 2 or 3 (**Table 13.1**), blubber color, characteristics, and thickness should be recorded. Focal blubber hematoma and edema may be indicative of blunt trauma and include damage to underlying tissues. Animals in robust body condition tend to ooze oil on incision of the blubber, which may appear homogenous and glistening. By contrast, for animals in suboptimal or catabolic states, it is not unusual to observe laminar pale to dark red discoloration of the basal half to third of the blubber layer, attributed to increased vascular perfusion and peripheral mobilization of fat stores (Koopman, Iverson, and Gaskin 1996). In addition, with animals in poor condition, there is little fat seepage, and the stroma may be more prominent, fibrous, and dull. Full-depth blubber with skeletal muscle tags should be collected either along the midlateral aspect of the thorax, between ribs 6 and 10, or from the dorsolateral aspect of the thorax, craniolateral to the dorsal fin, and then wrapped in aluminum foil and frozen (–20°C). The midlateral chest wall is the area of more rapid peripheral lipid mobilization of fatty acids from blubber, whereas the more dorsolateral region is typically targeted for biopsy of live animals in the field. In pinnipeds, midsternal blubber thickness and characteristics should be recorded. The skin can be used for genetic analysis and blubber samples to screen for fatty acid signatures, contaminant loads, and hormones.

In some animals that are autolyzed or frozen, dark red serous fluid may accumulate in the subcutis and abdominal and thoracic cavities, so exercise caution with interpretation of this fluid. The serosal surfaces of viscera should be closely scrutinized for possible fibrin tags that may suggest an acute peritonitis or pleuritis with serosanguinous effusion. In most cases, this fluid represents postmortem or freeze artifact.

Morphometrics

Prescribed morphometric measurements for different age classes and species have been well developed (**Table 13.2**), and for consistency, and cross-data analyses, an attempt to complete as accurate a recording as possible is recommended. Straight lengths of animals should be measured with a tape, and units recorded as centimeters. For pinnipeds, the animal is best laid in dorsal recumbency to flatten the spine. Small cetaceans are

Table 13.2 Marine Mammal Necropsy and Tissue Sampling Protocols in Chronological Order (from Most to Least Recent)

Reference and Source	Comments
Jepson and Deaville 2017a	Necropsy and sample collection protocols for stranded cetaceans in the United Kingdom.
Jepson and Deaville 2017a	Necropsy and sample collection protocols for mass stranded cetaceans in the United Kingdom.
Jepson and Deaville 2017b, 2010	Necropsy and sample collection protocols for stranded pinnipeds in the United Kingdom.
Plön et al. 2015	Region specific necropsy and sampling protocol for cetaceans.
Lane et al. 2014	Diagnosis of bycatch in small odontocetes in South African waters.
Raverty, Gaydos, and St. Leger 2014	Species-specific necropsy and tissue sampling protocol for killer whales (*Orcinus orca*).
Moore et al. 2013	Series of six multiauthor papers on gross and histopathologic diagnosis of anthropogenic traumas in cetaceans and pinnipeds, caused by boat strike, entanglement, entrapment, and gunshot.
Eros et al. 2007	Species and region specific for dugong (*Dugong dugon*). Stranding network, incident response, necropsy technique and sampling, diagnosis of cause of death.
Pugliares et al. 2007	Necropsy manual and sampling protocols for pinnipeds and cetaceans. Written for biologists.
Geraci and Lounsbury 2005	Most comprehensive guide for stranding response. All marine mammal taxa and international focus. Necropsy technique, sample necropsy reports, specimen and data collection, forensics and chain of custody, NOAA stranding reports and level A data collection, evaluation of carcasses for human interaction.
Hensley, Bossart, Ewing et al. 2005	Species- and organ-specific dissection and sampling manual for pygmy sperm whale (*Kogia breviceps*) heart.
McLellan et al. 2004	Species-specific necropsy and sampling protocols for right whale (*Eubalaena* spp.) necropsy.
Duignan 2000	All taxa in Australia and New Zealand. Necropsy procedures, sampling for diagnostics, listing of toxicology laboratories in Australia and New Zealand.
Read and Murray 2000	Photographs of typical bycatch lesions in small cetaceans.
Jefferson, Myrick, and Chivers 1994	Small odontocetes only. Necropsy equipment, illustrated dissection techniques, appendices with forms, sampling collection, and handling procedures. US focus.
Kuiken 1994; Kuiken and Hartmann 1991	Diagnosis of bycatch in cetaceans only. European focus. Collection of papers on gross and histological findings in bycaught cetaceans from NW Europe and the Black Sea.
Dierauf 1994	Pinniped forensic, necropsy, and specimen collection step-by-step guide.
Bonde, O'Shea, and Beck 1983	Species-specific protocols for West Indian manatee (*Trichechus manatus*).
Mazzariol and Centelleghe (undated)	Standard protocol for postmortem examination on cetaceans. Necropsy and sampling protocol adapted for cetaceans endemic to the Adriatic Sea, Italy.
Mazzariol, Cozzi, and Centelleghe (undated)	Handbook for cetacean strandings. Multiauthored collection of papers on necropsy of odontocetes and baleen whales, diseases of cetaceans in the Mediterranean, anthropogenic mortality, and response to live strandings.
Higgins and Noad (undated)	Necropsy and sample collection protocols for cetaceans, with a focus on Australia.

necropsied in lateral recumbency. Blubber thickness is typically measured from the epidermal–dermal junction to the base of the blubber, and in those cases where full-thickness measurements include the skin, the epidermal thickness should be recorded separately. For animals that are too large to record full circumferences, or where tape measures cannot be readily passed underneath the torso, doubling of measured half circumferences still provides valuable data. The degree of bloating should be noted. For baleen whales, measures of pleat lengths and their proximity to the umbilicus should be recorded along with the number, color, and maximum length of plate arcades. An intact baleen plate (longest present) may be collected, and in some species, earplugs should be harvested. These tissues can be analyzed for retrospective records of stable isotopes, reproductive and stress hormone levels, and contaminants (Trumble et al. 2013; Hunt et al 2016).

Photographs

Photographs may be recorded by a digital or digital single-lens reflex (DSLR) camera with either a 16–35 mm, 70–120 mm, or 60 mm macro lenses, and an appropriate scale with ruler, animal species, case log or identification number, and date should be included in the image. A wide-angle image to place

the position of the lesion in topographic context should be taken, followed by intermediate and higher-magnification photographs to record significant details. Photographs of normal anatomy are also valuable. A photographic log and brief description of the anatomic location and abnormality should be maintained. In some instances, strategically positioned stationary video recorders or use of head cams (e.g., GoPro) may be considered to document lesions and the necropsy procedure. Pathologists should be forewarned of video and voice recording or live streaming of necropsies to avoid the use of inappropriate language.

Dissection

With gross lesions, samples for histopathology should encompass both normal and abnormal portions of the tissue, and should be no more than 0.5–1.0 cm in thickness and up to 1–2 cm^2 on cut surface. Individual organs or anatomic location of sampled tissues for histopathology may be identified by use of attached laundry tags, cassettes, or labeled plastic bags. The volume of tissue–formalin should be 1:10 to ensure adequate fixation. Tissues with excess blood or sand should be rinsed with clean seawater prior to placement in formalin, and if the solution becomes too bloody, the formalin may be decanted and replaced. In the interest of safety, formalin may not be transported to some necropsy sites, and in these circumstances, samples may still be harvested and placed in a bucket, and then chilled until formalin is later added. Samples for fixation in RNAlater (for preserving RNA; Thermo Fisher Scientific) or glutaraldehyde (for electron microscopy) should typically be minced on a clean disinfected surface, and placed in solution, observing the same 10:1 fluid-to-tissue ratio. Consultation with the reference laboratory or investigators prior to tissue sampling and collection should ensure appropriate handling. In a diagnostic or laboratory setting, similar sampling strategies may be pursued.

The approach will be contingent to some extent on the position and postmortem state of the animal, work site access, and safety concerns. For animals in moderate to good postmortem condition and in dorsal or lateral recumbency, full-thickness transverse 0.5–1.0 m spaced parallel incisions of the blubber extending from the anus cranially to the back of the skull may be made. Horizontal cuts as high as possible along the flank of the animal will connect the transverse cuts and facilitate reflection of the blubber layer; in some instances, the fascia may be incised by a knife or flensing tool, or if necessary, traction may applied by hooks or chains attached to backhoes or other large equipment. As the blubber is reflected, the underlying tissues should be closely examined for abnormalities and appropriate tissues harvested. Once the blubber is completely reflected, it may be cut along the base and removed. On occasion, requests are made to have the blubber remain attached to facilitate cleanup and disposition of the carcass at sea, and caution should be exercised by prosectors, because the exposed surface can be slippery.

After the blubber has been removed, one team of prosectors may incise the abdominal wall, typically along the caudal margin of the ribs (costal arch), and dorsally, along the contour of the abdominal cavity, to the level of the rectum. Care should be exercised not to inadvertently cut internal viscera. As the abdominal wall is retracted, the cavity should be examined for any fluids or exudate, and appropriate samples collected in sterile containers for laboratory analysis and microbiology. Once the abdomen is fully exposed, the viscera should be photographed and assessed in situ for any abnormalities. In larger animals, routine tissues should be harvested and transferred to the dissection and sampling station (for subsampling). In smaller animals, intact viscera may be removed en block and placed on clean tarpaulins for examination and tissue processing. To minimize potential cross-contamination, if there are sufficient personnel, a separate team may be assigned to expose and examine the thoracic cavity. This is typically accomplished by transection of the costochondral junctions and incision of the intercostal muscles to release and remove individual ribs. In some instances, garden shears, a butcher's handsaw, or a chain saw may be used to facilitate access to the thoracic viscera; however, this should only be conducted after consultation to confirm that a cosmetic necropsy is not required. Chain saws should only be deployed where absolutely necessary and by skilled personnel wearing appropriate PPE. In some animals, to avoid transecting ribs, the diaphragm may be excised using an abdominal approach, with the dorsal and ventral mediastinum incised along its vertebral and sternal attachments, the trachea and esophagus transected at the level of the thoracic inlet, and then the heart and lungs retracted caudally from the thorax.

In larger animals, the heart and lungs may be examined in situ, and appropriate samples harvested through a "window" in the intercostal muscles. For specific disease entities, intravascular gas, pericardial fluid, or postmortem heart blood may be collected for analysis. Representative lung samples, particularly from the cranioventral, hilar, and caudal lung regions, should be collected. If possible, the skull may be disarticulated from the first cervical vertebra and brain samples collected either via the foramen magnum (an "apple core" approach) or by removal of part of the skull (occipital bones for dorsal access or through the roof of the mouth for ventral exposure). Due to increased awareness of acoustic-related trauma in cetaceans and pinnipeds and the specialized dissection and processing of extracted ears, a separate section with protocols is presented later in the chapter. If gas bubbles are identified in either the renal capsule or the mesenteric, epicardial, or pulmonary vasculature, detailed gas sampling protocols have been developed and are also included below.

To complete a full necropsy, the epaxial and hypaxial skeletal muscle should be removed and examined along with the vertebrae for possible physical injury or trauma, as well as degenerative changes and signs of infectious disease.

In large whales, when access is limited by the location or position of the whale, or time available to access the animal, individual portals or windows may be cut into the abdomen

and thorax to visualize organs and facilitate retrieval of samples ("window approach method"). Typically, these may be 1 × 3 m incisions into the blubber and underlying tissues immediately cranial to the rectum (to sample the colon, collect urine, and possibly evaluate gonads or kidneys); cranial limit of the abdominal cavity (to access the stomach and possibly collect small intestine, spleen, pancreas and liver); and mid to cranial third of the thoracic cavity (to expose the heart, lung, and regional lymph nodes). This technique is valuable for expedited collection of tissues to screen for infectious disease, harmful algal blooms (HABs), and histopathology.

In smaller animals with suspected trauma, radiographs and MRI or CT scans may prove invaluable in basic anatomic investigations, as well as to document injuries; however, these studies should only be pursued in consultation with radiologists, research scientists, and stranding response coordinators, when imaging facilities are readily accessible (Moore et al. 2009). In cases where overexposure or excessive noise is suspected as the cause of stranding, heads should be removed and ears exposed, recovered, and perfused with fixative as soon as possible for analysis. If there is evidence of blunt force, penetrating, or perforating injury, similar imaging studies may prove valuable in documenting the trauma and recovery of projectiles or bullets (Moore et al. 2013). Ultimately, the number and experience of the personnel involved with a necropsy will provide some guidance, in terms of what may be accomplished in a specific time frame.

Histopathology

Histologic examination provides insight into microanatomy and morphological changes associated with disease, pathogens, toxins, and trauma, and is vital for pathology. Tissues (see **Box 13.2**) collected should be no larger than 3 × 3 cm and ideally 0.5 cm in thickness. If larger samples are collected, numerous parallel cuts should be made in the tissue to improve fixative penetration. For standard evaluations, all tissues should be preserved in 10% buffered neutral formalin at a ratio of 1:10, tissue to fixative. For specific studies, other fixatives may be preferred, but maintaining standard histological fixation must become routine for marine mammal mortality investigations. All tissues from the same animal can be placed in the same

BOX 13.2 HISTOPATHOLOGY CHECKLIST

- ☐ Lung
- ☐ Trachea
- ☐ Heart
- ☐ Aorta
- ☐ Pulmonary artery
- ☐ Thymus
- ☐ Salivary gland
- ☐ Thyroid
- ☐ Tonsil
- ☐ Tongue
- ☐ Esophagus
- ☐ Stomach
- ☐ Duodenum
- ☐ Jejunum
- ☐ Ileum
- ☐ Colon
- ☐ Pancreas
- ☐ Spleen
- ☐ Liver
- ☐ Gallbladder
- ☐ Adrenal
- ☐ Kidney
- ☐ Ureter
- ☐ Urinary bladder
- ☐ Urethra
- ☐ Gonad
- ☐ Prostate
- ☐ Uterus
- ☐ Penis
- ☐ Eye (L/R)
- ☐ Ear (L/R)
- ☐ Brain
- ☐ Spinal cord
- ☐ Bone marrow
- ☐ Muscle
- ☐ Skin
- ☐ Blubber

Lymph nodes:

- ☐ Submandibular
- ☐ Cranial cervical
- ☐ Prescapular
- ☐ Axillary
- ☐ Tracheobronchial
- ☐ Hilar
- ☐ Gastric
- ☐ Hepatic
- ☐ Mesenteric
- ☐ Colonic
- ☐ Sublumbar
- ☐ Inguinal

OTHER:

container; however, specific lesions should be tagged or placed in labeled cassettes for identification. Laundry tags are useful labels to clip onto individual tissues. For each case, two labels should be used, one inside the container and one outside the container, and each container should only contain one case. Tissues should be allowed to fix for at least 48 hours before shipping. There are specific requirements for shipping tissues in formalin, since formalin is considered a hazardous substance.

Fetal, Placental, and Perinatal Examination and Sampling

In examining a fetus, if the amniotic and allantoic sacs are intact, these fluids should be sampled separately. The presence or absence of an umbilicus, its length, consistency, and whether it is fully or partially closed or healed should be recorded. For cetaceans, the fetal torso should be assessed for fetal skin folds and the numbers and orientation noted, photographed, or schematically recorded. The cranial biparietal width should be measured; this measurement has been used to gauge the stage of gestation. The dorsal fin may be curved to either side, and the direction should be recorded. The number and location of rostral hairs should be documented, as well as tooth eruption of the upper and lower arcades enumerated. Blubber thickness at the dorsal, midlateral, and ventral aspect of the mid thorax should be measured. Whether the eyelids are open and the extent of eye development may be important to estimate the stage of fetal development.

Dissection and exposure of the thoracic and abdominal cavities of the fetus can be approached as with general necropsy procedures for marine mammals. The internal and external aspect of the umbilicus should be examined for inflammation or infection, and the patency of the umbilical veins (to the liver) and artery (from the internal iliac arteries and closely apposed to the urinary bladder) assessed by insertion of a probe or other blunt instrument. If feasible, the patency of the ductus arteriosus (between the pulmonary artery and aorta) and foramen ovale (between the cardiac atria) should be determined. Also record the color and consistency of the lungs, and whether representative samples float or sink on immersion in formalin. The size and location of the thymus should be documented. The stomach contents can be described, and presence or absence of meconium in the colon recorded. As full a complement of fresh and formalin-fixed tissues should be sampled, with particular attention to recovery of stomach contents, brain, and lung for HABs and pathogen screening.

Forensic and Anthropogenic Mortality Investigation

A necropsy examination should always be conducted with an open mind, each animal being examined, samples collected, and the resulting information used to make a diagnosis. If anthropogenic trauma is suspected, the prosector may take additional samples and precautions to ensure that the case investigation withstands legal scrutiny, but the basic principles of pathological examination do not change. In a review of anthropogenic trauma and serious injury in pinnipeds and cetaceans; case definitions; circumstances associated with death; gross pathology; and histopathology of entrapment and drowning (bycatch), entanglement, blunt force injuries, sharp trauma, and ballistic projectiles were addressed (Moore et al. 2013). Another manifestation of anthropogenic activities described in marine mammals is gas bubble disease (GBD), particularly in beaked whales (Ziphiidae), and may be associated with deployment of military or industrial sonar (Jepson et al. 2003). Although the pathogenesis of GBD is distinct from acoustic injuries in the organ of Corti, both processes may be present and should be investigated in animals with a history of exposure, or gross lesions consistent with intravascular gas emboli and multisystemic hemorrhage. Mechanisms of GBD could also cause injuries to the auditory apparatus. For example, a decompression sickness–like syndrome in marine mammals might generate systemic gas and fat embolism, leading to auditory system pathologies (e.g., infarcts) that cause impairment of hearing or vestibular function (Fernandez et al. 2005).

Human interaction (HI) forms, documentation forms, entanglement response forms, and chain of custody forms are available and provide valuable templates to guide and record evidence of human interaction (Moore et al. 2013). It is imperative to accurately record carcass condition code and postmortem state. As these data and results from necropsy exams and laboratory studies may be subpoenaed and presented as evidence in court (see **Chapter 5**), chain of custody forms should be completed.

To diagnose anthropogenic trauma, detailed photographs, diagrams, and descriptions of gross external and internal wounds should be compiled, and if access to a radiology suite is available, imaging studies to document soft tissue and skeletal pathology and localize bullets or other projectiles should be pursued. In some larger animals, portions of the carcass such as the head or thorax may be removed to facilitate radiology. In many cases, bullets cannot be readily identified or retrieved by use of metal detectors. Bullets or other foreign debris should be recovered with care, using appropriate plastic instruments to minimize artifact, and then appropriately stored in a secured location once the postmortem exam is complete. Representative tissue samples for histopathology should be collected from the margins of the wounds, and soft tissues should be carefully removed from the carcass for close inspection of skeletal elements. Microscopic findings in skeletal muscle from animals with gross evidence of ship strike include hemorrhage; edema; and flocculent, discoid, granular, or hyalinized myocellular degeneration with contraction bands. In rare cases of prolonged entanglement in emaciated animals, protein casts have been identified in the lumina of

renal tubules, reinforcing the need to sample as complete a set of tissues as possible for histopathology.

Postmortem vitreous humor, or in fresh carcasses, heart or peripheral blood (serum), may be collected to quantify creatinine kinase and potassium levels, since increased values may substantiate the degree of muscle (or renal) injury (Sarran et al. 2008). Increased levels of the S100 B biomarker has also been reported in the vitreous humor of entangled northern fur seals (*Callorhinus ursinus*) and may prove valuable for suspect drowning cases (Roe et al. 2013).

Auditory Pathology

There is increasing concern and public awareness regarding the effects of man-made noise on marine mammals. Collection and examination of specimens from hearing structures is essential for the diagnosis of auditory pathology and to establish normal species, age, and sex-related anatomic baselines. Expanding our knowledge on the variation of inner ear morphology for various species is critical to the assessment of potential impacts of anthropogenic noise on marine mammals. Diagnostic modalities that have been developed and used to evaluate the integrity of the structures along the auditory pathway include computerized tomography (CT) scanning, gross dissections, electron microscopy, immunohistochemistry, and histological examinations (Ketten, Lien, and Todd 1993; Ketten 1997: Ketten, Cramer, and Arruda 1997; Seibel et al. 2010; Prahl et al. 2011; Morell et al. 2015, 2017; Ketten et al. 2016). When feasible, auditory evoked potentials (AEPs) may be collected antemortem in live stranded animals, to enhance our understanding of the neurophysiology (Houser and Finneran 2006) and pathology associated with auditory insults.

Because cochlear hair cells are very sensitive to autolysis, ear retrieval and processing should be initiated as soon as possible after death (**Figure 13.1**). When auditory injury is a strong possibility in either individual or mass strandings, efforts to disarticulate the head and initiate extraction of the ears should be a priority and expedited at necropsy. The head should be removed by incision of the atlanto-occipital joint and placed with the ventral side facing upward. The mandible and associated soft tissues should then be carefully examined and removed. A knife, number 12 curved scalpel, or chisel, can be used to remove the tympanoperiotic complex (TPC). For greater detail, refer to Ketten, Cramer, and Arruda (2007) for all cetacean species and Morell and André (2009) specifically for small odontocetes (**Figure 13.1a**). Care should be taken not to use excessive force or traction, which may fracture the periotic bone.

In terrestrial mammals, structural alterations of the organ of Corti (or hearing organ) and associated innervation are well documented in cases of permanent noise-induced hearing loss (Lim and Dunn 1979). Thus, it is crucial that the ultrastructural integrity of this organ and innervation are retained to screen for noise-induced hearing loss–related lesions and to establish baseline morphology. The optimal time frame for fixation for ears is within 5 hours postmortem. However, based on current protocols and available fixatives, ears can be collected and perfused up to 30 hours after death with sufficient retained detail for ultrastructural evaluation (Morell et al. 2017). To avoid damage or artifact to sensory epithelia, perfusion should be accomplished by slow, gradual injection of 10% neutral buffered formalin through the oval and/or round windows (Morell and André 2009).

Extraction of the TPC is followed by the following: (1) separation of the periotic from the tympanic bone (**Figure 13.1a**); (2) removal of the stapes by tissue forceps; (3) careful and superficial perforation of the round and oval window membranes with a needle; and (4) perfusion of the fixative slowly and progressively (with minimal pressure) through one window until the fixative seeps from the other window, using a soft catheter (gauge 14 IV catheter for small species, or the tip of a plastic pipette or a butterfly catheter for larger species) of the same diameter as the window's size and a 1 ml syringe (**Figure 13.1b**). Step 4 can be repeated starting from

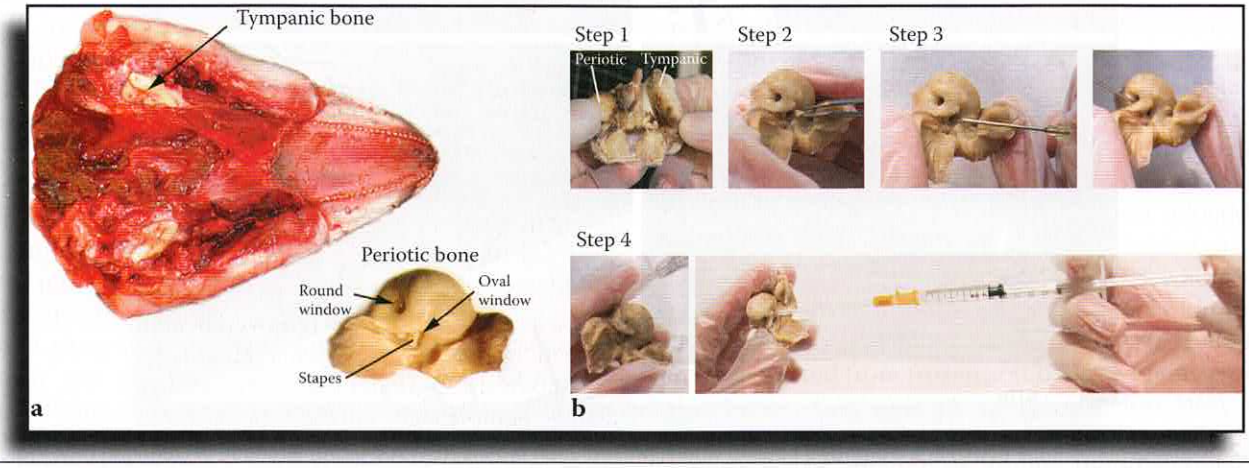

Figure 13.1 (a) Removal of the mandible in a harbor porpoise reveals the tympanoperiotic complex (TPC), from which the periotic bone can be extracted as shown. (b) Illustration of the perfusion steps on a periotic bone of a bottlenose dolphin. (Courtesy of M. Morell.)

the other window. It is crucial to follow the four steps in this order. After perfusion, the tympanic and periotic bones should be placed in a 1:10 volume of tissue to fixative and maintained in formalin for several days before shipping. If the postmortem interval is greater than 24–30 hours, if an infectious etiology, inflammation, or hemorrhage is suspected, or if the prosector is not experienced with the perfusion technique, the intact TPC can be immersed in the fixative solution for later decalcification and processing (Seibel et al. 2010; Prahl et al. 2011).

If imaging studies can be conducted immediately following death, CT scans of the head of smaller marine mammals can be undertaken before ear extraction (Ketten and Montie 2008). Ideally, the heads should not be removed as intravascular gas accumulation may occur within the brain and other soft tissues and mimic GBD. When field necropsy or ear extractions cannot be performed in a timely manner, the head may be frozen for later CT scanning and possible extraction/examination of the TPC. However, the integrity of the cells of the organ of Corti will be damaged by the freezing-thawing process, precluding ultrastructural interpretations. If frozen, gross examination and histopathology of the head or extracted TPC can still be undertaken; however, tissues will be compromised, and specimens should be thawed in fixative rather than in water or in air, to minimize autolysis.

In addition to the ears, other cranial tissues, such as acoustic fats, auditory canals, eyes (retina/sclera), and paraotic sinuses, should also be carefully examined, and representative samples collected for histopathological assessment.

Gas and Fat Embolism

The etiopathogenesis of this recently recognized condition, also known as decompression sickness (DCS) or gas bubble disease (GBD), is described in **Chapter 14**. GBD should be suspected and carcasses examined carefully if mortality is associated with any noise, or sonar or military activity, of if deep diving species, especially beaked whales, strand. Here we discuss the gross and histopathologic presentation and sampling for gas composition analysis, the key elements of diagnosis. Methodologies have been recently developed for in situ gas sampling and laboratory analysis that can distinguish between gas associated with putrefaction and gas primarily composed of nitrogen (Bernaldo de Quirós et al. 2011, 2012). A scoring system has also been developed that can help to distinguish between decompression-related gas lesions, iatrogenic air embolism, and putrefaction gases at necropsy (Bernaldo de Quirós et al. 2013a, 2013b).

On necropsy, acute and chronic presentations of GBD can be found. In the acute form, gas bubbles can often be seen in major organs and blood vessels. Acute gas embolism is often systemic, with bubbles and associated lesions in the brain, spinal cord, intestine (mesenteric veins), heart (blood

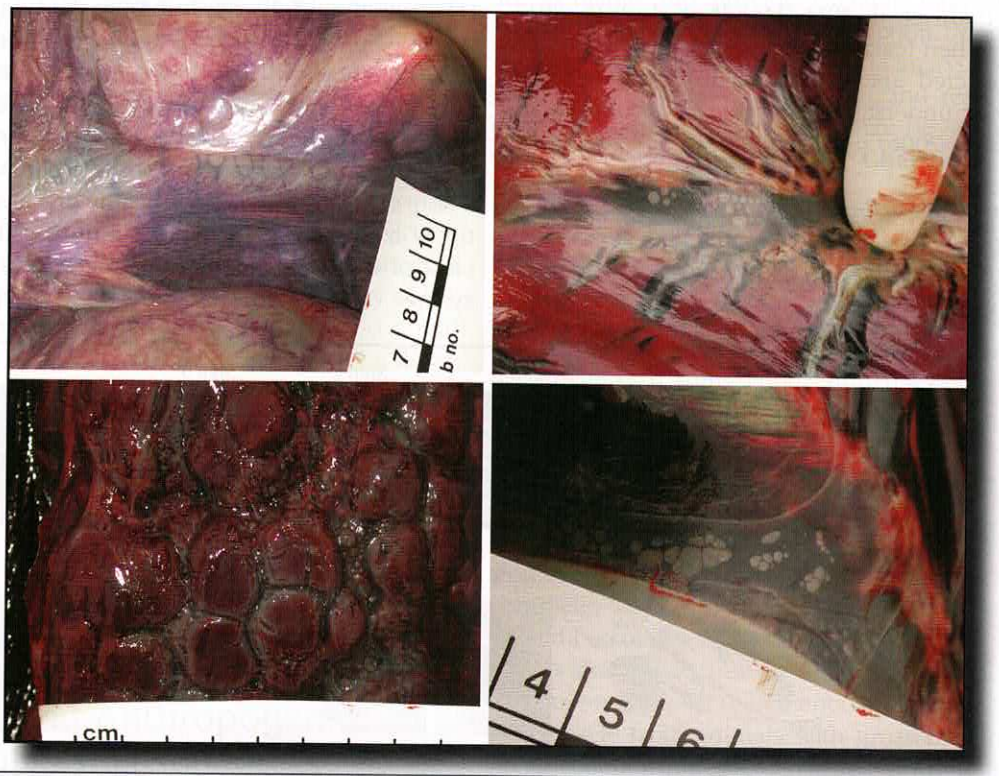

Figure 13.2 Gas bubble disease (GBD) in a Sowerby's beaked whale, *Mesoplodon bidens*, (SW2004/290) stranded in Cardigan Bay, Wales, UK, in September 2004. Clockwise from top left: bubbles in a mesenteric vein, bubbles in epicardial veins, bubbles in a mesenteric vein, bubbles in veins of the perirenal rete. (Courtesy of P. D. Jepson.)

vessels), renal (capsule/blood vessels and parenchyma), and liver (capsule and parenchyma). In severe cases (**Figure 13.2**), the quantity and distribution of gas bubbles and associated lesions can be extensive, and hemorrhages are often seen widely distributed in lipid-rich tissues, including brain and spinal cord. The chronic form of GBD can have a variable appearance. Large gas-filled spherical cavities of variable size (0.2–6 cm) have been seen in major organs, predominantly involving the liver, kidney, spleen, and gastric wall (Jepson et al. 2005; Bernaldo de Quiros et al. 2011). Bone lesions consistent with dysbaric osteonecrosis, seen in humans as a chronic consequence of decompression sickness, have been reported in sperm whales (*Physeter macrocephalus*; Moore and Early 2004). Histopathology of the gross liver cavities may reveal variable degrees of pericavitary fibrosis. In addition, intrahepatic microcavitations, typically 50–750 µm in diameter (consistent with gas emboli), within distended portal vessels and sinusoids may be associated with hepatic tissue compression, hemorrhages, fibrin/organizing thrombi, and foci of acute hepatocellular necrosis. Gross renal cavities (2–10 mm diameter) may be associated with acute and chronic arterial gas emboli–induced renal infarcts (Jepson et al. 2005).

Fat embolism can accompany severe and acute systemic gas embolism (Fernandez et al. 2005). In gas and fat embolic syndrome, fat emboli are most readily observed in lung tissue, widely distributed within the small veins and capillaries. Fat emboli have also been found within the veins and lymphatics of the epidural retia surrounding the spinal cord, in the subcapsular sinuses of lymph nodes, and occasionally in small medullary veins in the kidney. It is possible to use special stains to show fat embolism microscopically (e.g., Oil-Red-O on frozen sections or osmium tetroxide postfixation technique in formalin-fixed tissues) in lung and other tissues (Fernandez et al. 2005).

All gas bubbles and associated lesions (both grossly and microscopically) should be photographed. On initial opening of the body wall into the carcass, before any samples are taken, the subserosal blood vessels of the intestinal wall and mesenteric veins should be thoroughly examined for presence of bubbles. The vessels associated with the subdermal sheath, kidney, and renal capsule should also be examined for bubbles and gas emphysema in the capsule. Bubbles may also occur in the CNS (brain/spinal cord), heart (blood vessels), kidney (capsule/blood vessels and parenchyma), and liver (capsule and parenchyma).

The amount of gas present in veins and tissues can be evaluated retrospectively using photographs taken during the necropsy, and semiquantified by assigning a gas score to different vascular locations, as well as to the presence of subcapsular gas (emphysema), defined as macroscopically visible gas found beneath the capsule of body organs (e.g., kidneys). Vascular locations commonly used for gas scoring cetaceans are subcutaneous, mesenteric, and coronary veins, as well as the lumbocaudal venous plexus. A scoring rubric has been developed: grade 0 is no bubbles, grade I represents a few bubbles, and grade II represents abundant bubbles. Subcapsular gas is also scored: grade 0 is no subcapsular gas, grade I represents the presence of subcapsular gas in one or two organs, and grade II represents widely distributed emphysema through the body. The summation of gas score (0 to II) in the different vascular locations and tissues gives the total gas score, ranging from 0 to 10 (Bernaldo de Quiros et al. 2011, 2012).

In Situ Gas Sampling, Transport, and Analysis of Gases

Before gas samples are taken, it is important to establish a relationship with the laboratory that will conduct the gas analysis, including the optimal conditions for sample storage and transport method, prior to shipment for analysis. Samples to be shipped by air must be in a pressure-tight vessel to avoid pressure change during flight. To minimize masking by putrefaction gases, necropsy and gas sampling must be performed as soon as possible after death, and preferably within 12 hours. Bernaldo de Quiros et al. (2011, 2012) found that vacuum tubes, insulin syringes, and an aspirometer were reliable tools for in situ gas sampling, storage, and transportation without appreciable loss of gas and without compromising the accuracy of the analysis. Gas analysis is usually conducted in the laboratory by gas chromatography, where high percentages of N_2 (~70%) and CO_2 (~30%) are indicative of nitrogen supersaturation (decompression sickness).

Genetics

Knowledge of the species, as well as the specific population from which an animal came, is critical for interpreting data collected from live or dead animals. Although many different tissues have been used for genetic analysis, skin and liver are the most commonly collected tissues. White blood cells, muscle, gonads, teeth, and bone have also been collected from carcasses, and white blood cells or skin biopsies are typically collected from live animals (**Table 13.3**).

Genetic analyses require only a small sample; the recommended sample size for collection is 0.5 cm^2 soft tissue cut into small strips for preservation. One milliliter of blood, whole teeth, or a piece of bone has also been collected for genetics. The best method of preservation depends on the tissue collected. Soft tissue, such as skin, is best preserved in 5–20% DMSO in saturated salt solution at 1 volume of tissue to 10–20 volumes of preservative. The solution containing the tissue should then be frozen for long-term storage. DNA can be extracted from frozen soft tissue without preservative, but it is more difficult, particularly if nuclear DNA (e.g., microsatellites) is to be analyzed. Alternative methods include fixation in 80% ethanol, or drying. Blood samples are best frozen.

Table 13.3 Protocols for Specimen Collection for Life History Data

Analysis	Sample	Taxonomic Group	Collection Site	Code	Size	Fixative
Genetics	Epidermis Muscle Leukocytes Bone Teeth	All	Varies with tissue used	1-5	1 cm × 0.5 cm, cut in strips, whole bone or teeth; 10–20 ml of whole blood	5–20% DMSO solution, 80% EtOH, saturated salt, or freeze
Age	Teeth	Odontocetes	Mandible (left)	1-5	Whole tooth with root intact; whole jaw	70% ethanol or freeze whole
		Phocids	Premolar (postcanine)	2-5		
			Canine, incisor	1		
		Otariids	Canine	2-5		
		Sirenians (Dugongs)	Canine	2-5		
		Odobenids	Mandibular canine	2-5		
		Ursids	First premolar either jaw	2-5		
		Otters	First premolar either jaw	2-5		
	Bone	Sirenians (Manatees)	Tympanoperiotic bone (periotic dome)	2-5		Fixed 10% buffered neutral formalin or freeze
		Cetaceans	Tympanic bullae Vertebrae Metacarpals	2-5		
		Pinnipeds	Metacarpals	1-5		
	Earplugs	Mysticetes		2	Whole, intact	10% buffered neutral formalin.
	Baleen	Mysticetes		2-5		Dried
	Eyes	All	Lens	2	Whole	Frozen
	Claws	Otters	Claws	1-4	Whole	Whole in 10% glycerin or in 70% ethanol
Prey	Stomach contents Feces		Stomach Feces	1-3	Whole volume of stomach contents, when possible	Piscivores—freeze intact; herbivores—10% neutral buffered formalin
Reproductive status	Gonads Uterus Serum		Both ovaries Both testes Other organs as noted	1-4	Whole ovaries; whole testes with epididymis or full cross and longitudinal sections of testis; whole or portions of uterus	Fixed with 10% neutral buffered formalin in normal position; can be frozen if no fixative available Take morphometrics before fixing
Morphometrics				1-5		

Stomach Contents

Evaluation of stomach contents is important both for diagnostic evaluation and for assessment of prey selection. Stomach content analyses are time-consuming efforts and should be performed by experienced personnel; however, collection and storage of contents are easy to perform in the field. Stomach contents may include otoliths, macerated prey flesh, skeletal remains, parasites, foreign bodies, and vegetation. Fish otoliths are one of the most commonly used structures for prey identification. In addition to the shape and characteristics of an otolith being species-specific, the size of the otolith is proportional to fish size, allowing for evaluation of size class of prey, as well as caloric intake of the marine mammal.

In small animals, the stomach may be tied off at both ends and frozen intact for later examination, although freezing may limit pathologic and parasitologic examinations. Ideally, the stomach should be opened when fresh, the mucosa gently

flushed with saline, and the contents (including the washings) frozen or fixed for later evaluations. The type of preservation of the contents will depend on the expected diet of the various taxa of marine mammals. Buffered neutral formalin fixation may dissolve the otoliths of some prey fishes; therefore, formalin fixation should not be used for preserving stomach contents from fish-eating marine mammals. These contents instead should be frozen or fixed in alcohol. Stomach content samples from plant-eating marine mammals (e.g., manatees; *Trichechus Manatus*) should not be frozen, since the freezing of sea grass and algae causes fragmentation of the cells, making identification very difficult (Eros et al. 2007). Instead, stomach contents from herbivorous animals should be preserved in 5–10% neutral buffered formalin or 80% ethanol at a ratio of 1:1 or 2:1 (Eros et al. 2007). Subsamples for toxicology or biotoxins are collected from the stomach contents of fresh carcasses when they are first opened, and the subsamples frozen for later evaluation. If the stomach is opened, fresh parasites can be collected (see below), and the mucosa examined for pathology. Freezing and thawing may limit identification and interpretation of gastrointestinal pathology and parasites. Foreign bodies need to be documented and photographed, and ingested marine debris or fishing gear is saved, whenever possible.

Age

Currently, age is estimated primarily from counts of growth layers deposited in several persistent tissues, primarily teeth, and less often, bone (**Table 13.3**). Saving teeth or other tissue for aging from known-age animals (from the wild or captive situations) is also important, because these tissues are used to validate the interpretation of growth layers for specific taxa. At times, relative measures of age, such as tooth wear, pelage or skin color, or fusion of cranial sutures, which allow individuals to be placed in age groups, are helpful. Age class or maturation status may be estimated using body size/length (Stevick 1999), fusion of epiphyses, pelage color, or reproductive parameters. Use of body size as a rough estimate of age, however, requires that a growth curve has been generated for that species from allometric models that fit size-at-age data for a large number of specimens for which age was known or estimated from growth layers.

Growth layers (or growth layer groups; Perrin and Myrick 1980) in teeth have been used to estimate age for odontocetes and pinnipeds (Hohn et al. 1989; Oosthuizen 1997), since they were first associated with age by Scheffer (1950). For small cetaceans, growth layers are counted primarily in dentine, although for a few species (e.g., the Franciscana [*Pontoporia blainvillei*] and beaked whales), cement is better. For pinnipeds, growth layers are counted in both dentine (the yellowish, calcified tissue that makes up the bulk of all teeth, harder than bone, softer than enamel) and cement (thin bone-like material covering roots of teeth, softer than dentine). Canines are best for dentinal counts, but in very old animals, the pulp cavity may be occluded, and cement must then be used. Cement is best counted in postcanines (Klevezal 1996). Incisors can be safely extracted from live animals, but these reduced teeth have small layers, and age tends to be underestimated by significant amounts in old animals (Bernt, Hammill, and Kovacs 1996). For dugongs (*Dugong dugon*), the tusk (incisor) or canine can be used (Eros et al. 2007).

For a number of species, notably manatees and baleen whales, teeth cannot be used for age estimation. Manatees have an indeterminate number of molars that are constantly lost and replaced throughout life, and no tusks. Baleen whales have no teeth. Fortunately, annual growth layers do occur in the tympanoperiotic (auditory) bones of manatees (Marmontel et al. 1996) and baleen whales (Klevezal 1996). For each species, the location on the bone with the maximum number of layers must be found; in other regions, resorption of early-deposited layers results in an underestimate of age. For all bones, growth layers occur in periosteal bone, and generally, the maximal number of layers occurs where the periosteal bone is thickest.

In balaenopterid whales, earplugs also have been used for age estimation (Lockyer 1984; Kato 1984). These structures are actually a horny epithelium formed in layers on the external surface of the tympanic membrane of the external auditory meatus. In addition to numbers of growth layers, a change in the morphology of the growth layers from irregular layers (immature) to regular layers (mature) has been seen in some species and is thought to indicate the transition to maturation (Thomson, Butterworth, and Kato 1999). Specific chemicals and hormones can be extracted from individual layers of cetacean earplugs (Trumble et al. 2013). Chemical signals, specifically amino acid racemization, have been used for dolphins and small and large species of whales (Bada, Brown, and Masters 1980), including, most recently, fin whales (*Balaenoptera physalus*) and bowhead whales (*Balaena mysticetus*; George et al. 1999). Age is estimated as a function of the proportion of D and L isomers of aspartic acid in the lens of the eye.

Accurately and precisely counting the annual layers depends greatly on the tissue and techniques used. For example, Hohn and Fernandez (1999) found that stained sections allow more accurate estimates of age in bottlenose dolphins (*Tursiops truncatus*), and Stewart et al. (1996) found a similar result for ringed seals (*Pusa hispida*). Validation of the growth layer deposition rate for specific species has been done using teeth from known-age animals (Hohn et al. 1989) or teeth from animals that had been exposed to tetracycline at a known point in time. Tetracycline binds with calcium and is incorporated into active tissues (e.g., teeth and bone) within 48 hours of administration (Frost 1983). Under visible light, tetracycline-marked bone and/or teeth exhibit yellow-brown coloration. Under fluorescent light, marked bone exhibits a yellow-gold fluorescence.

Teeth are the best tissues to be collected from odontocetes and pinnipeds for aging. For small odontocetes, it is standard to collect teeth from the middle of the left mandible; six to eight teeth should be collected if the skull is not going to be

kept. When the left mandible is not available, center teeth from the other mandible or from the maxilla are satisfactory, with the emphasis being on large, straight teeth. For pinnipeds, the best tooth to collect may depend on the relative age of the animal (juvenile, adult, old adult). To be certain that an accurate age can be obtained may require collecting several teeth, including canines and postcanines. However, for manatees and large whales, the ear bones should be collected. Because growth layers are integral to teeth and bone, these tissues are not sensitive to most means of storage. They can be frozen in plastic bags or vials, stored in 70% ethanol, or cleaned of soft tissue and dried. Short-term storage in formalin is acceptable. They also can be soaked in water to facilitate cleaning prior to preservation or further analyses. Care should be taken that the teeth are not damaged or broken during extraction. In certain field situations, it may be more practical to collect and save the entire mandible or skull with teeth intact for later extraction and processing. If earplugs can be collected, they are to be handled gently (because they are fragile) and fixed in formalin (Lockyer 1984). For estimation of physical maturation, physeal fusion of bones, such as vertebrae or carpal/metacarpal bones, may be evaluated from frozen or dried samples. Radiographs of flippers may assist with maturation determination, and whole flippers can be frozen for later examination. Eyes should be collected and frozen for extraction and analyses of the lenses from condition code 2 animals (George et al. 1999). Claws can be frozen or kept dry.

Reproductive Status

Gonads should always be collected even when decomposition is advanced. When possible, fresh weights and measurements should be taken. Both ovaries need to be collected, especially for small cetaceans, which have unequal ovulatory patterns. Whole ovaries should be fixed in 10% buffered neutral formalin when possible, or frozen if no fixative is available. They should not be cut or subsampled for histology until after they have been examined in gross (whole and thick sections) for corpora (see **Chapter 10**). Gross examination of the uterus is performed for detection of pregnancy and whether the uterus appears to have been distended sufficiently to suggest that a pregnancy has occurred in the past. In small animals, the uterus with ovaries can easily be preserved intact in 10% buffered neutral formalin, but care should be taken that the uterus is fixed in a natural position rather than folded into a small container. If the uterus is large, it should be weighed (when possible), measured, and examined. Gross examination and measurements should include myometrial wall thickness, cervix, internal diameter of uterine horns, length of uterine horns, any lesions, fetal presence–size–position, parasites, and associated lymph nodes. Representative tissue samples should be collected in 10% buffered neutral formalin.

Testes and epididymides are to be removed intact. If possible, testes and epididymides are to be weighed separately. Testis length (ensuring to exclude the epididymis), width, and depth are important parameters, with mass and length of primary importance, especially if the testis cannot be collected whole. Because studies have shown no significant difference in size between the left and right testes, both testes do not need to be collected. The opposite testis can be used to collect a subsample for histological examination, fixing all tissues in 10% buffered neutral formalin. When whole testes cannot be collected, an alternative is to collect a complete 1-cm-thick cross section from one testis and a complete longitudinal section from the other, and fix these sections in formalin, preferably in a flattened position. They can later be rolled for storage in a jar. Samples from any reproductive tract lesions are also fixed in 10% neutral buffered formalin. From fresh animals, blubber, serum, feces, and urine may also be obtained. These can be used to determine reproductive hormone levels to correlate with gross findings.

Contaminants

For assessment of organic pollutant levels that are lipophilic, blubber, milk, blood, and liver are typically analyzed; for assessment of elements, kidney, liver, blood, and skin (epidermis) are also used. Target organs, if known, also need to be collected for complete evaluation of effects and residue levels in marine mammals. Blubber is collected from specific sites (depending on the taxon), and samples should be full thickness, as some species have both vertical and horizontal stratification in blubber. A minimum of 20 grams (ideally 100 grams) should be collected of each tissue type with a clean stainless steel or Teflon knife. Tissues are collected in clean glass jars or in Teflon bags and stored at temperatures less than −80°C. For a limited time, tissues can be stored at −20°C; however if long-term storage is expected, the tissues should be stored at −80°C, and if tissues are to be archived, the tissues should be stored in liquid nitrogen. When collecting tissues, ensure that the specimens or collecting instruments are not in contact with aerosols of insect repellent, smoke, exhaust fumes, petroleum fumes, or other chemical contaminants that may alter the chemical analyses of the tissues (see **Chapter 15**). Tissues may also be contaminated during the necropsy by gut contents or blood, thereby altering the actual measured values. Whole blood, serum, or plasma has been used for chemical analyses; a minimum of 10 ml of selected matrix should be collected and stored frozen in clean glass jars or Teflon jars/bags. Because storage of tissues or fluids in plastic can alter the chemical analyses for some compounds, tags and collection forms must note the use of such. Whenever tissues are collected for pollutant analyses, a field collection description should include the conditions under which the tissues were collected, and the instruments and materials used for collection, processing, and storage.

Polycyclic aromatic hydrocarbons (PAHs) are rapidly metabolized in marine mammals, so the likelihood of finding

circulating levels or tissue levels indicative of acute exposure is low. However, serum, bile, and liver can be assessed in acute exposure cases. From those taxa that have gall bladders, collection of bile is performed by withdrawing fluid from the gall bladder using a syringe or by clamping off and excising the gall bladder. Once the gall bladder is excised, bile may be poured into a dark cleaned glass container. This is the preferred collection method for pinnipeds. Collection of bile from cetaceans is more difficult but can be accomplished by withdrawing bile from the large hepatic duct. Bile should be protected from light and can be placed in dark jars or in clear glass containers that have been wrapped in foil. These containers should be stored frozen at −80°C.

Dermis or liver can be used for assessment of cytochrome P4501A as a surrogate for PAH exposure; however, several compounds increase levels of cytochrome P4501A (see **Chapter 15**).

Infectious Diseases

Sampling for infectious diseases is reviewed in each chapter on groups of infectious agents (**Chapters 17–21**). Abnormal tissues, fluids, and body organs sampled for histology can be tested for presence of infectious diseases and disease agents using direct visualization, culture, and molecular techniques. Sampling a piece of each tissue selected for histology, storing at −80°C, and fixing in 90% alcohol and RNA later are useful contingencies when the etiology is unknown.

Conclusions

Postmortem examination of marine mammals uses essentially the same techniques developed for pathology of domesticated species and wild mammals, but the need for clear diagnosis of anthropogenic trauma as well as collection of samples for life history, ecology, and physiological studies guide the use of more specific protocols in certain cases. A suite of protocols are available on the internet, and practitioners should become familiar with the protocols most suited to species likely to be encountered in their region, as well as associated management issues of concern, before conducting necropsies.

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