NETCET MONITORING STRANDING PROGRAM

Cetacean strandings create an important opportunity for the gathering of much needed knowledge on natural and human-induced mortality of cetacean populations, and provide an available source for precious additional information, among other things, on the biology, pathology, toxicology and population genetics of the concerned species. According to the ACCOBAMS Agreement, stranding networks should exist in the area, each of them having various degrees of the extent of their spatial and temporal coverage, efficiency, and institutional involvement. One of the main target in the NETCET project is to ameliorate efficiency of these network in order to have a best picture of cetaceans status in the Adriatic basin.

In order to implent this action, several tools were foreseen within the project itself and, in particular:
1. a common database;
2. common information to be collected and exchange;
3. scientific networking;
4. specific training;
5. the adoption of common protocols and procedures;
6. the production of a periodic report of strandings trends in the Adriatic with analysis of temporal and spatial distribution and main causes of death with special emphasis to those related to human activities;
7. the creation of a regional task force in order to face environmental emergencies and epidemic outbreak.

Every Adriatic Countries should adopt and endorse this monitoring tool which are harmonized with existing national and ACCOBAMS recommendation and legislation. The following paragraph give a brief explanation of each tool giving also reference documents produced in the NETCET project. Since no existing recommendation were found for sea turtles, what stated for cetaceans was implemented also for sea turtles considering national and local rules.

1. Common Database
Despite the existence of a Mediterranean Cetaceans stranding database (MEDACES) and national ones, Adriatic Databases dedicated to sea turtles and cetaceans were considered necessary to give a regional picture of strandings occurring in the basin allowing spatial and temporal analyses. These tools give to the partners and other interested stakeholders the possibility to study the phenomenon. Database will be update in the next 5 years periodically (every three months). In case of unusual mortality events they could be used for monitoring in real time.

Reference:
2. Common information to be collected and exchange
Sharing of information is necessary for transboundary cooperation. A minimum set of information to be collected and exchange has been decide at the beginning of the project using a questionnaire. Minimum data set both for cetaceans and sea turtles are:
- timing
- location with GPS coordinates
- animal information: species, sex and size
- dead or alive
- results of veterinary examinations/action: cause of death, releasing information
- contacts of the reporter
- other

3. Scientific networking
The creation of specific forum, mailing lists of the NETCET project and contact lists allow to scientist and organization involved stranding monitoring to exchange protocols, information and updates.

Reference
- List of Scientific Organization involved in Technical Network (annex I)

4. Specific training
A specific action dedicated to training was organize in the NETCET project: training courses and workshops were dedicated to veterinarians, rescue centers and volunteers involved in monitoring program in order to enforce cooperation and diffuse common procedure and knowledge within the Adriatic area. In order to enforce these actions, specific handbooks dedicate to sea turtles and cetaceans have been printed and distributed to most relevant stakeholders.

Reference
- Handbook for cetaceans strandings (Italian and English Version)
- Handbook for sea turtles strandings (Italian and English Version)

5 Common protocols and procedures
Specific protocols (sea turtles and cetaceans necropsy protocols) and procedures (handling procedure for sea turtles and cetaceans) were prepared and diffuse during trainings and workshops dedicated to main stakeholders involved in cetaceans’ and sea turtles’ strandings monitoring program. Protocols and procedure should be periodically revised.

Reference
- Standard protocol for post-mortem examination on sea turtles (annex II)
- Standard protocol for post-mortem examination on cetaceans (annex III)
- First aid and management procedures of sea turtles (annex IV)
- Stranded cetaceans handling and first aid. (annex V)
- Stranded sea turtles handling and first aid. (annex VI)

6. Periodic reporting
Data collected during the monitoring program should be periodically reported to Institutions and general public (every three years). In particular, these reports should focus on strandings distributions, trends and causes identifying critical areas, periods and main threats for cetaceans and sea turtles in the Adriatic, with special regards to mortality related to human activities. Data on causes of death should be obtained by necropsy reports produced using the standard protocols (point 5) and be assessed using an evidence-based approach. Graphs and maps are welcome to better explain data and compare with previous periods.

7. Emergency Task Force
In case of environmental emergencies and unusual mortality events, a regional task force has been proposed to share information, personnel and equipment. This Adriatic Task Force define specific roles and communication system with special emphasis to international cooperation within specific agreements. Every country should identify national focal points and reference labs to be involved in case of emergencies.

Reference
- Proposal for the creation of an Adriatic Emergency Task Force.
ANNEX 1

LIST OF SCIENTIFIC ORGANIZATION INVOLVED IN THE TECHNICAL NETWORK

All the Scientific and Technical Institutions and Organizations listed below are directly involved in the NETCET Project or have been included in NETCET scientific and training activities. Contact person reported are actively involved in mailing list and discussions on technical and scientific items.

1) Istituto Zooprofilattico Sperimentale delle Venezie - Italy (Dr. Anna Toffan and Dr. Francesco Pascoli)
2) Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna - Italy (Dr. Silva Rubini and Dr. Paola Massi)
3) Istituto Zooprofilattico dell'Abbruzzo e del Molise - Italy (Dr. Nicola Ferri and Dr. Gabriella di Francesco)
4) Istituto Zooprofilattico Sperimentale dell'Umbriae delle Marche - Italy (Dr. Gianni Perugini and Dr. Stefano Gavaudan)
5) Istituto Zooprofilattico di Puglia e Basilicata - Italy (Dr. Antonio Petrella)
6) Dept of Veterinary Medicine, University of Teramo - Italy (Prof. Giovanni di Guardo)
7) Dept of Veterinary Medicine, University of Bari - Italy (Prof. Antonio di Bello, Dr. Olimpia Lai and Dr. Nicola Zizzo)
8) Istituto Zooprofilattico del Piemonte, Liguria e Val d'Aosta - Italy (Dr. Alessandra Pautasso and dr. Cristina Casalone) - Italian Reference Center for Cetaceans' Diseases.
9) Dept of Comparative Biomedicine and Food Science - Italy (Dr. Sandro Mazzariol and Dr. Lisa Poppi)
10) Dept. of Biology of the University of Padova - Italy (Prof. Maria Berica Rasotto and dr. Gabriella Marin)
11) Fondazione Cetacea Onlus - Italy (Dr. Valeria Angelini and Sauro Pari)
12) Associazione Benessere Animali Onlus - Italy (Dr. Giordano Nardini)
13) WWF Italia - Italy (Dr. Paolo Casale)
14) Centro Studi Cetacei Onlus - Italy (Dr. Vincenzo Olivieri and Dr. Massimiliano Pennelli)
15) Museum of Venice - Italy (Dr. Nicola Novarini)
16) ISPRA - Italy (Dr. Caterina Fortuna and Dr. Otello Giovanardi)
17) Dept. of Veterinary Medical Science, University of Bologna - Italy (Dr. Mauro Delogu and Dr. Annalisa Zaccaroni)
18) WWF - Protected Marine Area of Miramare - Italy (Dr. Francesco Zuppa)
19) National Institute of Oceanography and Sperimental Geophysics - OGS - Italy (Dr. Valentina Tirelli and Dr. Stefano Querin)
20) Regional Agency for Prevention and Ambient - A.R.P.A. of Friuli Venezia Giulia and Emilia Romagna - Italy (Dr. Massimo Celio and Dr. Carla Rita Ferrari)
21) Slovenian Environment Agency - ARSO (Agencija Republike Slovenije za Okolje) - Slovenia
22) State Institute for Nature Protection - Croatia (Dr. Ana Maricevic and Dr. Jasna Jeremic)
23) Blue World Institute of Marine Research and Conservation - Croatia (Dr. Drasko Holcer and Dr. Peter Mackelworth
24) Faculty of Veterinary Medicine, University of Zagreb (Dr. Martina Gomercic and Dr. Tomislav Gomercic)
25) Marine Educational Center - Croatia (Dr. Karin Gobic Medica)
26) Herpetofauna Albanian Society - Albania (Dr. Vilma Piroli)
27) Association for Protection of Aquatic Wildlife of Albania - Albania (Dr. Sajmir Beqiraj)
28) Croatina Veterinary Institute - Croatia (Dr.
29) Institute for Marine Biology - Montenegro (Dr. Mirko Jurovic)
30) Museo Civico di Storia Naturale di Milano (Dr. Michela Podestà) and Dept. of Biology and Biotechnology of University of Pavia - Italy - Italian Strandings Database
STANDARD PROTOCOL FOR POST-MORTEM EXAMINATION ON SEA TURTLES

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PREFACE

Sea turtles are marine reptiles with peculiar anatomy, physiology and habits. Considered "threatened" by the IUCN, a lot is still unknown about these animals. Fortunately, in the last few years an increasing number of research facilities have developed growing interest in these animals, but still very little bibliography is available for a systematic data collection. Some protocols have been developed for the dissection of sea turtles, but clear schemes for sample collection are lacking even now.

Drawing inspiration from similar protocols in other endangered wild species, this protocol is a mix of previous procedures and our experience, providing a practical approach for the conduction of routine data and sample collection. Furthermore, this protocol is designed to improve knowledge about diseases of sea turtles in the wild by providing guidelines to complete more comprehensive necropsies and disease testing.

Using standardized necropsy protocols is important to facilitate comparison of data among stocks or population. Screening for specific pathogens (i.e., Herpesvirus and Mycobacterium chelonii) is of increasing importance for assessing population health and the presence of potential zoonoses. In some cases, negative results are as meaningful as positive ones.

It is our hope that once people on the Adriatic Sea realize the need to learn more about diseases of sea turtles, this protocol will also increase available knowledge through the execution of complete postmortem necropsies.
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1. EXTERNAL EXAMINATION

1.1 Signalment

1.1a - Species Identification: there are several elements that identify the species of a sea turtle, but the most efficient are the number and disposition of costal scutes and prefrontal scales. Subjective variations in these patterns are possible.

- Caretta caretta: 5 lateral scutes, 2 pairs of prefrontal scales,
- Chelonia mydas: 4 lateral scutes, 1 pair of prefrontal scales
- Dermochelys coriacea: no scutes - 5 dorsal ridges, no scales

<table>
<thead>
<tr>
<th>Species</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caretta caretta</td>
<td>5 lateral scutes, 2 pairs of prefrontal scales</td>
</tr>
<tr>
<td>Chelonia mydas</td>
<td>4 lateral scutes, 1 pair of prefrontal scales</td>
</tr>
<tr>
<td>Dermochelys coriacea</td>
<td>5 dorsal ridges, no scales</td>
</tr>
</tbody>
</table>
1.1b - Collection of Morphometric Data: different scheme are proposed from different scientific groups. The most important are: weight, curved carapace length (CCL), head length and width (HL and HW), total tail length (TTL), and vent-tip length (VTL). For measurements, use scales, measuring tape and/or callipers. See Annex I.

1.1c - Sex Determination: to determine the sex of a sea turtle is not easy, because adult females differ little in external morphology from large, immature males. Typically, females have a short tail and the cloacal opening (vent) is located roughly half way between the tip of the tail and the plastron's anal scute. Within the cloaca, the genital papilla remains small as the clitoris on the floor of the cloaca. Adult males are characterized by a long tail with the cloacal opening near the tip, and strongly curved claws on the second digit.

In younger animals (< 60 cm CCL), the sex of the animal may be known for certain only during the necropsy through accurate examination of the gonads. However, in hatchling and very young subjects (< 30 cm CCL) it may be very difficult to establish if gonads are ovaries or testicles due to the underdevelopment of the reproductive system and the temperature-dependent sex determination. In hatchlings, histologic analysis will help.

Regarding sex determination, species differences are not noteworthy.

1.2 Condition code.

Before initiating the necropsy, carcass condition must be determined. Preferably, necropsies are performed on fresher carcass (within 48 hours of death); however, environmental conditions can greatly impact condition code.

If human interaction is suspected or forensic data are of value, necropsies should be performed irrespective of tissue quality. Carcasses are classified in one of five code categories depending on the level of decomposition.

**Code 0:** Alive or just died (< 2 hours post mortem).

**Code 1:** Fresh carcass (< 24 hours post mortem). Normal appearance, usually with little scavenger damage, fresh smell, minimal eyes drying, eyes clear, carcass not bloated.

**Code 2:** Moderate decomposition. Bloated carcass with characteristic mild odour. Head: integral or with partial loss of skin; eye: sunken or liquefied; tail: present or absent; limbs: integral; carapace and plastron: integral.
**Code 3**: Advanced decomposition. Collapsed carcass with strong odour. Head: complete loss of skin; eye: liquefied tail absent; limbs: partially exposed skeleton; carapace and plastron: partial or total loss of skin.
Subgroup "a": distinguishable internal organs.
Subgroup "b": liquefied internal organs.

**Code 4**: Mummified carcass or partial carcass. Incomplete carcass; skull: visible; carapace: broken with separation of parts.

### 1.3 Nutritional condition (1).

The body condition of a sea turtle is not easy to assess. A good idea of the body condition can be obtained by looking at the roundness of the neck, the depression of the eyes and of the axillary and inguinal regions. Nevertheless, in a fresh carcass (condition code 0 and 1), sunken eyes and neck are also signs of a dehydrated animal, and flaccid axillary area may be considered muscle cachexia. Furthermore, in condition code 2 axillary and inguinal areas up to the tail are severely bloated, so can be impossible to establish if there are fat or muscles in these positions.

Then, the correct nutritional code is assignable only by looking the ventral fat deposition after plastron removal. Also in this case, very important to keep in mind that fat deposition is strongly
dependent on sex, size, season and reproductive stage.

1.4 Integument.

The first note to take from an external examination is the presence of injuries, scars, deep parallel cuts or absence of scutes on the carapace or plastron. Equally important is to note the number, type, size and disposition of (if present) ectoparasites (barnacles, leeches) and algae on the carapace, plastron, visible skin and mucosae. Loss of some scutes is normal in condition code 3 and 4. The examination should include the investigation and description of the eyes, mouth, vent, and skin.

When examining the eyes, look for discolouration, injuries, or discharge, and for ectoparasites on or near the eyelids.

Document any lesions in the mouth or of the tongue: fracture of the jaw, cuts (especially if linear and deep), and foreign bodies. Pay attention to the normal detachment of the rhamphotheca in condition code 4 and sometimes 3.

Look for parasites and discharge around the vent. Obtain samples of abnormalities for histology, microbiology and molecular investigations. Sample parasites for parassitology diagnosis.

Thoroughly examine and document any scars, ulcerations, erosions, wounds on the carapace and plastron, especially if regular in shape and in disposition (linear, parallel..). Make note of the size (length x width x depth/height), shape, colour, texture, location and distribution of all abnormalities.

Remove about 30 g of scutal cheratin from the carapace for genetic analysis (frozen and DMSO) and small pieces of skin with lesions for histology. Be sure that all morphometric measurements have been completed before collecting these samples.
2. INTERNAL EXAMINATION

2.1 Plastron removal.

To perform a sea turtle necropsy, plastron removal is the first step. With the carcass placed on its back (if necessary, stabilize the carcass with paper, cloth, beach towel or make a hole in the sand), cut with a scalpel all around the plastron. Pay attention to the direction and the depth of the cut, particularly between marginal and inframarginal scutes. In this position the coelomic cavity is particularly close (especially in summer, when there is less fat), whilst under the cranial and caudal part of the plastron, the subcutis and muscles are present between the plastron and body cavity. Pulling the plastron upwards cut the underlying muscle (anterior part of the ventral muscles - pectoralis major) with cuts parallel to the plastron in a cranio-caudal direction. At approximately a quarter of the plastron length (more or less central where the gular scutes meet the humeral scutes) note the white connective strong connection of the plastron with the acromion processes of the scapula. Cut it as close as possible to the plastron to avoid damaging the pericardial sac and/or the heart. Regarding the second half of the plastron, a large portion of the underlying surface is covered by muscle (caudal part of the ventral muscles - rectus abdominis). As before, continue cutting close to the plastron: in the central part of this area, usually fat deposition is found between the plastron and underlying peritoneum, but for cachectic animals or during the summer this could be very thin.

Once the plastron has been removed a surface covered by muscles and fat is observable. The muscles are the large pinnate (feather-shaped) “chest” muscles used for swimming, and the fan-shaped pelvic muscles that were attached to the plastron. Fat is usually partially liquefied, shiny, perfuse and with holes; the colour is olive-green or brownish-orange, depending on the type and percentage of minerals inside, resultant from feed. Fat covers entire ventral area.

During the execution of all these procedures, development, thickness, texture and colour of muscles and thickness and colour of fat deposition must be noted, as well as presence of haemorrhages, haematomas, oedema or other focal/multifocal lesions over the entire field. Obtain muscle samples for histology and contaminants and fat samples for toxicology and fat composition analyses.
2.2 Nutritional condition score (2).

Once the plastron is removed, it is possible to assign the correct nutritional condition score. It is very important to keep in mind that fat deposition (localization, thickness and colour) is strongly dependent on sex, size, season (feed, water temperature, time spent in feed activities) and reproductive stage.

**Score 1 - Excellent:** Integral or partially liquefied adipose tissue covers entire ventral area. Hepatic lipidosis is also present.

**Score 2 - Very good:** Integral or partially liquefied adipose tissue covers entire ventral area.

**Score 3 - Fair:** Integral or partially liquefied adipose tissue covers peripheral parts of ventral area.

**Score 4 - Scarce:** Integral or partially liquefied adipose tissue is present only in limited peripheral parts of ventral area.

**Score 5 - Not valuable:** Mummified carcasses.
2.3 Removal of the Scapula and approaches to the viscera.

To uncase the viscera, perform a sagittal cut through the pectoral muscles, gently separating all the muscular masses including the scapula of both sides from beneath the pericardium and peritoneum, rotating the entire flippers anteriorly outwards. In this way, the brachial artery, axillary vein and brachial plexus are observable and by reversing the flippers under the carcass, it is definitively stabilized. Cut the peritoneum sagittally (evaluate the fat thickness if possible) and observe location, size and colour of the liver and gastrointestinal tract respectively and if there are exudates or fibrin depositions. Cut the pericardium and observe the heart and great vessels. Look for lesions and exudates and describe them (localisation, appearance and quantity). In any case, if fluids are present, measure them and make cytological smears from them.

For an overall view of the main organs, gently move the gastrointestinal tract to see the lungs, gonads, bladder and adrenal glands above it and identify retroperitoneal renal masses. The pancreas and spleen are observable along the intestine.

2.4 Heart and Great Vessels.

The heart, great vessels, thyroid gland and thymus are found in the space beneath the muscles located between the two acromion and the coracoid processes. Particularly the pericardium is very thin and near the muscular masses, so it will usually be damaged during the scapula removal.

Before handling the heart, observe and describe the pericardium. Note if there is excessive fluid and describe the characteristics. Also, note the thickness of the tissue.

Trim away the pericardium and observe the epicardium (external surface of heart) in situ. Note
size, colour, and texture of each heart structure: sinus venosus, two large (right and left) atria and a ventricle, and the great vessels: two aortas (right and left) and a pulmonary trunk. The ventricle is attached to the pericardium via a fibrous connective tissue cord called the gubernaculum cordis. To remove the heart, it is necessary to cut the sinus venosus and the gubernaculum cordis. Whilst opening the heart chambers and the great vessels, examine them closely to look for parasites or lesions caused by them. Sample the ventricle, the atria and the aorta for histology.

2.5 Thyroid, Thymus, Parathyroid and Ultimobranchial Body.

**Thyroid** - The thyroid sits in the ventral neck, often coated with a thin layer of yellow fat, which masks it very well. In fresh carcasses is gelatinous, liquefied in other carcass conditions. Note the size, shape, colour and texture. Sample for histology, microbiology and molecular investigations.

**Thymus** - The thymus is a large, lymphoid organ formed by two structures. In decomposed carcasses, it become more similar to the surrounding fat. Note the size, shape, colour and texture. Sample for histology, microbiology and molecular investigations.

**Parathyroid and Ultimobrachial body** - These two glands are distinguishable only histologically. They are very difficult to identify even in a really fresh carcass. They are located along the carotid and ventral cervical arteries. If found, sample for histology.

2.6 Liver and Gallbladder.

The liver is the largest visceral organ and surrounds the heart. In fresh carcasses and in some periods of the year it is not unusual to find the liver pale or orange and soft (severe lipidosis). Examine the surfaces of the liver and note colour pattern, texture and size of the lobes. Examine the parenchyma of the liver and the gallbladder by performing several
parallel cuts through the tissue. Again, note the internal colour and texture and look for parasites. Sample for contaminants, histology, microbiology and molecular investigations.

2.7 Oesophagus.

The oesophagus is located left of the trachea, then it passes through the tracheal bifurcation and it takes position above the lungs and under the heart and liver (with the carcass on its back). To expose the oesophagus, cut the skin of the ventral neck from the position of the intergular scute (previously removed) to the jaw, separate the underlying muscles and move aside the trachea. Cut the oesophagus and let it slide through the tracheal bifurcation; then, draw it away with the entire gastro-intestinal tract (after tying the rectum). Using scissors, cut through the entire length of the oesophagus from the cranial part to the stomach; observe the serosal and mucosal surfaces of the oesophagus and the presence, quantity and type of feed and parasites. Note, colour, texture and contents. Sample for histology.

2.8 Gastrointestinal tract.

After the oesophagus, the digestive apparatus is formed by stomach and intestine. The stomach forms a moderate dilation in the digestive tube. To avoid contaminating the remaining tissues or losing contents, it is
necessary to tie off both ends of every part of the digestive tract (stomach, small and large intestine) prior to cutting. With some twine, tie a tight, secure knot at the location of the attachment of the esophagus to the stomach. A second piece of twine can be tied just below the base of the pylori where the small intestine begins. Unlike in mammals, the intestine is poorly differentiated between small and large intestine, so a third knot may be tied at approximately half the length of the tube. The last knot has to be tied at the rectum just before the bladder. Examine the external surface of the gastrointestinal tract for discolouration and lesions. Separate each section and empty the contents into individual identified containers through a sieve. Thoroughly examine to look for fluid, mucus, whole or partially digested fish, fish bones, parasites, and foreign objects; in particular note the presence of marine litter and/or fishing devices (hooks, fishing lines, fishing nets). Save all foreign objects for human interaction documentation. Once empty, cut the wall to examine the mucosa; note the colour and texture, look for ulcers, parasites, areas of discolouration and other abnormalities. Sample for histology and for toxicological analysis.

2.9 Pancreas.
The pancreas is attached to the mesentery along the duodenum, past the stomach, and half surrounds spleen with its caudal pole. Remove the pancreas from the cavity by detaching it from the connective tissue and duodenum. Note the size, shape, colour, and texture of the surface. Cut into the parenchyma and note changes in colour or texture, or look for very small discoloured nodules, referable to multifocal hyperplasia or eventually parasitic egg infestation. Take samples for histology investigation.

2.10 Spleen.
The spleen is located in the mesentery at the end of the pancreas. Note size, shape, colour and texture of both the surface and the parenchyma of the spleen. Sample for histology, microbiology and molecular investigations.

2.11 Urinary Bladder.
The bladder is found centrally, just under the pelvis and above the rectum (with the carcass on its
Before removing the bladder from the body, be sure to clamp the bladder using a haemostat in order to retain urine. The anatomical position of the bladder sometimes allows the entrance of foreign materials: faecal materials, sand, eggs in females and also parasites. Open the bladder in a small container, collecting all the content: very often there are parasites inside. Note colour, consistency, amount of urine and presence of parasites; refer the content to the parasitologist. Examine the bladder internally by cutting along the length of the organ to expose the mucosal surface. Note colour and texture of the mucosa. Sample the cranial tip of the bladder for histology.

2.12 Trachea.
To expose the trachea, cut the skin of the ventral neck from the position of the intergular scute (previously removed) to the jaw and separate the underlying muscles. Using scissors, cut through the entire length of the trachea from the bifurcation up to the apex of the throat. Examine the mucosa and identify and describe contents (froth, fluid, blood, colour, etc.) and look for parasites. Sample for histology.

2.13 Lungs.
The lungs are attached to the carapace and vertebral column. They are easier to investigate directly in the body. Removal the lungs may be difficult and time consuming, versus the possibility to touch and cut lungs and bronchi and observe texture and colour and look for parasites directly in situ.
Cut the trachea opening where it bifurcates into two bronchi, and then follow the bronchus that enters the lung and continues. Next, in the lung, make serial cuts parallel to the long axis of the body to examine the parenchyma. This is best done with a long knife using a single sweeping cut in order to avoid tearing or serrating the lung tissue. During the cut, note whether fluid, froth and/or parasites are present and describe amount, colour, etc. Examine the parenchyma and pleural surface: note colour pattern and texture. Sample for histology, microbiology and molecular investigations.

2.14 Adrenal Gland.

The _Caretta caretta_ has a single adrenal gland, located centrally, close to the dorsal aorta, between the kidneys. To remove the adrenal, grasp and pull the tissue away from the body wall and cut the surrounding connective tissue. Before sectioning, measure (LxWxH) and weigh the adrenal. Note size, shape, colour and texture of the tissue. Sample adrenal for histology investigations.

2.15 Urogenital system.

The urinary and genital systems form a unique system with the terminal of digestive tract (cloaca). During the necropsy, the gonads are easy to examine. **Ovaries and oviducts** - The ovaries and oviducts change a lot in size and composition with age and between breeding and nonbreeding seasons. Obtain measurements (LxWxH) and
weight of each ovary. Examine the size, and the developmental stage. Sample each ovary for histology.

Testes - Testes are attached by the mesorchium to the peritoneum. Obtain measurements (LxWxH) and weight of each testis. Examine the size, shape, colour and texture externally and internally. Sample each testis for histology.

2.17 Kidneys.

The left and right kidneys are retroperitoneal and attached to the caudal roof of the carapace. To detach kidneys from the carapace may be difficult and time consuming. The easiest way to examine them is to perform a deep single sweeping cut and examine the internal surface and the capsule. Look for eventual oedema, other lesions or fluid; note colour, thickness and opacity. Note the size, shape of both kidneys.

Sample for contaminants, histology, microbiology and molecular investigations.

SKULL

2.18 Removal of the Brain.

The brain is the most fragile and easily disrupted tissue in the entire body, thus extreme care must be taken when removing the brain from the skull. First the head must be detached from the body to safely remove the brain. Do so by cutting behind the supraoccipital crest (at the start of the neck) down to the joint between the skull and cervical vertebrae, and then completing the cut ventrally. Once separated, firmly stabilize the head, then, using a Stryker saw or a hacksaw, make a parasagittal cut through the head from one of the nostril backwards along the major axis.
of the head and uncase the brain and the large subdural space containing cerebral spinal fluid, the third ventricle and cerebral aqueduct ventral. Alternatively, cut away the top of the head with a minimally inclined cut from the dorsal margin of the eyes towards the middle of the supraoccipital crest. In this way, the tubular brain appears white and elongated in the braincase, covered by the two-layer meninges and immersed in the clear cerebral spinal fluid. Olfactory tracts to the nose, optic lobes, cerebral hemispheres and cerebellum may be seen. The two round, lobed structures visible laterally to the brain and dorsally and posteriorly to the eyes are the salt glands. It will take some practice to successfully cut the head without penetrating the brain, also because different sea turtles differ slightly in brain morphology and location, even between cheloniids, and even more when compared to Dermochelys.

Using a scalpel, gently cut the meninges, sample cerebral spinal fluid with a sterile needle and syringe for cytology and culture, work under the brain to sever each cranial nerve. Inversion of the head often allows the brain to gently descend into the palm of your hand.

2.19 Examination of the Brain.
As stated before, the brain is the most delicate tissue in the body and will fall apart if handled excessively. Observe the external surface of the brain and note symmetry of each distinct structure (right and left cerebral hemispheres, cerebellum, and brain stem) while noting the colour, texture and presence of exudate or lesions. Vascular congestion can be a result of positioning or post mortem lividity. The best way to preserve the brain is to cut it sagittally and put an entire half brain in formalin and sample the other half brain for microbiology and molecular investigations. During the cut, note symmetry, colour, texture and the presence of lesions.

2.20 Pituitary Gland.
Once the brain has been removed, the pituitary gland can be seen immediately under the crossover of the optic nerve. The organ is within a bony recess and is usually small. It can be extracted after incision through the overlying dura by lifting it out using small forceps and a scalpel blade. Sample for histology and other priority testing.

2.21 Salt Glands.
The salt (lacrimal) glands are dorsal and medial to the eyes, and are responsible for removal of excess salt from the body.
3. SAMPLING PROTOCOL

3.1 Life History
Code 1, 2 and 3, ideal; 4 and 5 limited.

- **Age determination** –
  - Humerus, should be kept frozen.

- **Reproductive Status** –
  - Gonads and Uterine samples fixed in 10% NBF.

- **Feeding Habits** –
  - Stomach contents can be collected into a sealable plastic bag or jar, freeze.
  - Carapace keratin, for stable isotopes should be kept frozen. What the animal has been eating recently.

3.2 Genetics
Codes 1; 2-4 are suitable. Only one of the following needs to be collected, unless there are specific requests.

- **Internal organs, Muscle, Bone** - Better to freeze tissue samples, in case the tissue is used for something other than genetics. Genetic tissue samples can be fixed in DMSO saturated with NaCl.

- **Blood** – can only be collected from Code 1 and 2 animals. Minimum amount is ~10 ml; 50-100 ml is optimal for DNA studies.

3.3 Microbiology
Code 1 and 2 are ideal; 3 limited; 4 and 5 useless. Take separate samples for bacteriology and virology. Lesions should be sampled from several distinct locations, include normal tissue with the infected tissue sample.

- **Bacteriology** – Avoid freezing samples for bacteriology if avoidable. Refrigerate samples at 4°C. Freezing at −70°C is preferable to decomposition.
  - External samples can be taken with a swab from the eye, and vent. Culture swab in a bacterial transport medium.
  - Internal samples can be taken from the intestine, heart, kidneys, lungs, liver, spleen, bone with marrow, and tissues showing pathological changes. Culture swab in a bacterial transport medium or 6 x 6 cm sample placed in a sterile container.
  - Fluid samples can be taken from the pleural fluid, peritoneal fluid, urine, blood, fluid from abscesses. Store in appropriate aerobic or anaerobic vial.

- **Virology** – Refrigerate samples at 4°C.
  - External samples use a sterile swab dipped in viral transport medium. Take samples from the eye and vent. Place swabs in the vial that contains the viral transport medium.
  - Internal samples can be taken from the CNS tissues, lungs, liver, spleen, kidneys, tissues with pathological changes, intestinal contents. 6 x 6 cm sample placed in a
sterile container.

- Fluid samples from pleural fluid, peritoneal fluid, pericardial fluid, urine, blood from heart. Store in sterile container.

### 3.4 Parasites

Samples taken from Code 1-4 animals are suitable for examination.

- **Barnacles** – first fix in 10% NBF, for no more than 24 hrs, then transfer to 70% EtOH.
- **Copepods & Amphipods** – place directly into 70% EtOH.
- **Nematodes (roundworms)** – Fix in GAA for 5-10 minutes first if possible. Otherwise use 70% EtOH or 10 % NBF. If formalin is used, fix only for a few hrs. Then transfer to 70% glycerin alcohol.
- **Trematodes (flukes/flatworms)** – Dead or alive, fix in AFA for up to 3 days, transfer to 70% EtOH. Do Not use glycerin alcohol.
- **Cestodes (tapeworms)** – Fix for 5-10 min. in AFA solution and water, 4:1 ratio. Transfer to 70% EtOH. Include cestode head when removing from the host, if necessary cut host tissue.
- **Acanthocephalans** – Fix in AFA for up to 24 hrs. Then transfer to 70% glycerin alcohol.

### 3.5 Histopathology

Code 1, 2 and 3 are ideal. Rare / endangered species should be thoroughly sampled. Lesions, fractures, lacerations, and gunshot wounds of any code should be sampled in this manner.

- Tissues should be preserved in 10% NBF.
- Tissue samples should be no larger than 3 x 3 cm and approximately 1 cm in thickness.
- Ideally, histo samples should be cassetted and placed into a labeled jar for the appropriate Institution / researcher. Individual requests should be noted and tracked.
- Samples of gross lesions should include abnormal and normal tissue.

### 3.6 Contaminants / Biotoxins:

(organochlorines, heavy metals)

Code 2 is ideal, 1 and 3 is limited; 4 and 5 questionable to useless.

- **Biotoxin Analysis** – Code 2 animals only. Collect stomach contents, liver and/or kidney tissue. Freeze.
- **Contaminant Analysis** – All of the samples should be frozen in plastic zip lock bags and/or wrapped in aluminum foil.
  - Skin and subcutaneous tissue, muscle, liver, kidney, and brain (if possible include cerebrum and cerebellum).
4. BIBLIOGRAPHY


5. ANNEX I

MORPHOMETRICAL DATA

<table>
<thead>
<tr>
<th>BASIC MEASURES</th>
<th>COMPLEMENTARY MEASURES</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 = TL</td>
<td>8 = PL</td>
</tr>
<tr>
<td>2 = CCL</td>
<td>9 = PW</td>
</tr>
<tr>
<td>3 = SCL</td>
<td>10 = TTL</td>
</tr>
<tr>
<td>4 = CCW</td>
<td>11 = VTL</td>
</tr>
<tr>
<td>5 = SCW</td>
<td>12 = FoL</td>
</tr>
<tr>
<td>6 = HL</td>
<td>13 = HiL</td>
</tr>
<tr>
<td>7 = HW</td>
<td>14 = CaTT</td>
</tr>
</tbody>
</table>

TL: total length
CCL: curved carapace length notch to tip
SCL: straightline carapace length notch to notch
CCW: curved carapace width
SCW: straightline carapace width
HL: head length
HW: head width
PL: plastron length
PW: plastron width
TTL: total tail length
VTL: vent-tip length
FoL: foreleg length
HiL: hindleg length
CaTT: tip of carapace-tip of tail
6. ANNEX II

GUIDELINES FOR SEA TURTLES NECROPSIES

1. ANAMNESIS AND SIGNALMENT (WHERE? SPECIES?)
2. EVALUATION OF PRESERVATION STATUS (SEE PRESERVATION SCORES TABLE)
3. COLLECTION OF MORPHOMETRICAL DATA
4. DISSECTION:

   4.1 external examination of the animal (EXTERNAL WOUNDS? SKIN ABNORMALITIES? EXTERNAL BLEEDING?)
      \(\rightarrow\) **PARASITOLOGY**: external parasites research by simply observation (how many barnacles? Leeches?)

   4.2 removal of plastron

   4.3 evaluation of nutritional score (SEE NUTRITIONAL SCORES TABLE)

   4.4 removal of scapula and associated muscles (HAEMORRHAGES? PALENESS?)

   4.5 medial cut on neck skin: observation of thyroid gland and thymus (SIZE? COLOUR? SHAPE?)

   4.6 opening of pericardium; observation of great vessels, removal and dissection of the heart (PERICARDIAL EFFUSIONS? EPICARDIAL OR MIOCARDIAL LESIONS?) – weight
      \(\rightarrow\) **PARASITOLOGY**: blood flukes research in the heart and great vessels or related lesions on vessels wall

   4.7 opening of celomic wall and general observation of the cavity (CELOMIC EFFUSIONS? TOPOGRAPHY? COLOUR ABNORMALITIES? SPREADING LESIONS?)

   4.8 liver evaluation and removal (COLOUR? CONSISTENCE? SIZE? GROSS LESIONS?) - weight
      \(\rightarrow\) **PARASITOLOGY**: research of liver parasites by sedimentation exam

   4.9 removal of whole gastrointestinal tube (EXTERNAL WALL LESIONS? INTUSSUSCEPTIONS?) - length ; opening of the entire tube (GASTRIC ULCERAE? INTESTINAL WALL LESIONS-PETECHIAE? CONTENT-ANY FOREIGN BODY? WHICH AND HOW MUCH FOOD?)
      \(\rightarrow\) **PARASITOLOGY**: research of adult parasites by simply observation of content + sedimentation exam; research of eggs by qualitative coprologic exam from rectum content; research of Cryptosporidium oocysts from smears of rectum content by modified Ziehl-Neelsen stain

   4.10 pancreas and spleen evaluation (COLOUR? CONSISTENCE? SIZE? GROSS LESIONS?) - spleen weight

   4.11 external observation, removal and opening of urinary bladder (INTERNAL WALL LESIONS?)
      \(\rightarrow\) **PARASITOLOGY**: research for adult parasites in bladder content (usually Trematoda) by simply observation and/or sedimentation exam.

   4.12 opening of the remaining part of esophagus (FOOD? FOREIGN BODIES? WALL LESIONS?)
      \(\rightarrow\) **PARASITOLOGY**: research of parasites in the esophagus (usually Nematoda at junction with stomach)

   4.13 observation of lungs (CONSISTENCE? COLOUR? GROSS LESIONS?); opening of the trachea (WALL LESIONS? ANY FLUID INSIDE?)
      \(\rightarrow\) **PARASITOLOGY**: research for adult parasites in trachea and bronchi by simply observation after longitudinal opening of their cartilage

   4.14 genital organs observation and removal (WHICH GONADS?? OVARIC FOLLICLES PRESENT? EGGS?)

   4.15 kidneys observation (GROSS LESIONS?) - weight

   4.16 eyes observation and removal (GROSS LESIONS?)

   4.17 brain and salt glands removal (cut away the top of the head)
5 SAMPLE COLLECTION

- **histology**: all lesions
- **bacteriology**: pericardium, lungs, intestine, kidneys, lesions
- **virology**: lung, liver, kidney, brain and/or peripherical nerves, intracardiac blood clot
- **toxicology**: liver, muscle, kidney, skin, gonads
- **genetics**: visceral organs, skin, scutes keratin, muscle, bone

## PRESERVATION CONDITION SCORE

<table>
<thead>
<tr>
<th>Score</th>
<th>Preservation status</th>
<th>Description</th>
<th>Practicable exams</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fresh carcass</td>
<td>Death occurred in the previous 24 hours</td>
<td>histology, cytology, virology, microbiology, parasitology, pollutants, biotoxins, genetics</td>
</tr>
<tr>
<td>2</td>
<td>Moderate decomposition</td>
<td>Head: integral or with partial loss of skin;</td>
<td>histology (limited), virology, microbiology, parasitology, pollutants, biotoxins, genetics</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Eye: sunken or liquefied;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tail: present or absent;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Limbs: integral;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carapace and plastron: integral;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bloated carcass</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Advanced decomposition</td>
<td>Head: complete loss of skin;</td>
<td>histology (limited), parasitology, pollutants (limited), genetics</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Eye: liquefied;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tail: absent;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Limbs: partially exposed skeleton;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carapace and plastron: partial or total loss of skin;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collapsed carcass</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>a) internal organs: still distinguishable</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>b) internal organs: liquefied</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Mummified carcass or partial carcass</td>
<td>Incomplete carcass;</td>
<td>genetics</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Skull: visible;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carapace: broken with separation of parts</td>
<td></td>
</tr>
</tbody>
</table>

## NUTRITIONAL CONDITION SCORES

<table>
<thead>
<tr>
<th>Nutritional status</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excellent</td>
<td>Integral or partially liquefied adipose tissue covers entire ventral area. Hepatic lipidosis is also present.</td>
</tr>
<tr>
<td>Very good</td>
<td>Integral or partially liquefied adipose tissue covers entire ventral area.</td>
</tr>
<tr>
<td>Fair</td>
<td>Integral or partially liquefied adipose tissue covers peripheral parts of ventral area.</td>
</tr>
<tr>
<td>Scarce</td>
<td>Integral or partially liquefied adipose tissue is present only in limited peripheral parts of ventral area.</td>
</tr>
<tr>
<td>Not valuable</td>
<td>Mummified carcasses.</td>
</tr>
</tbody>
</table>
7. ANNEX III

NECRO REPORT

<table>
<thead>
<tr>
<th>Event Info</th>
<th>Animal Info</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strand Date:</td>
<td>Species: C. caretta</td>
</tr>
<tr>
<td>Recovery Date:</td>
<td>C. mydas   D. coriacea</td>
</tr>
<tr>
<td>Euthanized / Died</td>
<td>Sex: M F (after necropsy)</td>
</tr>
<tr>
<td>Date &amp; TOD:</td>
<td>CCL: _________ cm</td>
</tr>
<tr>
<td>Necro Date &amp; Time:</td>
<td>Weight: _______ Kg</td>
</tr>
<tr>
<td>Storage Prior to Necropsy:</td>
<td>Condition at Stranding: 1 2 3 4</td>
</tr>
<tr>
<td>Stranding Location:</td>
<td>Condition at Necropsy: 1 2 3 4</td>
</tr>
<tr>
<td></td>
<td>Human Interaction: Yes / No</td>
</tr>
<tr>
<td>Lat/Long: _________N/_________W</td>
<td># Animals: __________</td>
</tr>
</tbody>
</table>

CARCASS DISPOSITION:

HISTORY:

COMMENTS:

Necropsy Observations: Please note general observations of color, condition, textures, etc. even when utilizing NA= not applicable, NE= not examined, NSF= no significant findings, NVL= no visible lesions. List weights (g) next to each organ examined.
EXTERNAL EXAM

Carapace/Plastron/Skin (color, condition):

Wounds/Scars:
mark the figure

Lesions:
mark the figure

Parasites (what, where):

Vent:

Mouth (tongue, ulcers)/ Mucous membranes (color):

Eyes (discharge, color, ruptures):
**NUTRITIONAL CONDITION**

<table>
<thead>
<tr>
<th>Fat deposition:</th>
<th>Excellent 1</th>
<th>Very good 2</th>
<th>Fair 3</th>
<th>Scarce 4</th>
<th>Not valuable 5</th>
</tr>
</thead>
</table>

Coelomic cavity (fluid content, colour, quantity):

**MUSCOLO/SKELETAL SYSTEM**

Muscle (haemorrhages/haematomas/discoloration/atrophy):

Ventral muscles:

Posterior muscles:

**CIRCULATORY SYSTEM**

Pericardium:

Heart:

Vessels (parasites):

**LIVER and GASTROINTESTINAL SYSTEM**

Liver (color, congestion, lesions, size) and gallbladder:

Esophagus (parasites):
Stomach (content, ulcers, mucosa, parasites):
weight full ___________ weight empty__________

Intestine (contents, ulcers, parasites):

Pancreas:

Spleen:

RESPIRATORY SYSTEM

Trachea:

Bronchi (parasites):

Lungs (color, condition, edema, congestion, consolidation, granulomas, emphysema, lesions, parasites):
(R)

(L)

URINARY/REPRODUCTIVE SYSTEMS/ADRENAL

Bladder (parasites):

Testes / Ovaries: Immature / Mature with eggs / follicles / corpora albicans
(R) Lx W x H cm:

(L) Lx W x H cm:
### Kidneys (color, condition):

(R)

(L)

Adrenal: L x W x H cm

### OTHER GLANDS

**Thyroid:**

**Thymus:**

### SKULL

**Brain:**

**Salt glands:**

### Differential Diagnosis from Gross Exam
### 1. GENERAL INFORMATION

<table>
<thead>
<tr>
<th>N. ID</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>CCL</td>
</tr>
<tr>
<td>Examiner</td>
<td></td>
</tr>
<tr>
<td>Cause of death</td>
<td>Date of death</td>
</tr>
<tr>
<td>Location of necropsy examination</td>
<td>Date of exam</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Video</th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photo</td>
<td>YES</td>
<td>NO</td>
</tr>
</tbody>
</table>

| Condition Code |
|---|---|---|---|
| 1 | 2 | 3 | 4 |

**Video**

- **Note:**
  - **ND:** Not Determined
  - **NE:** Not Evaluable

### 2. EXTERNAL EXAM

**a. Body condition**

- **Emaciated**
- **Not emaciated**
- **ND**
- **NE**

**b. Sings of fishing net or lines**

(indicate if YES, NO, ND, NV for each area and in the positive case describe the lesion)

- **Head**
- **Mouth**
- **Flipper sx**
- **Flipper dx dx**
<table>
<thead>
<tr>
<th>2. EXTERNAL EXAM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>c. Presence of fishing nets on the animal</strong></td>
</tr>
<tr>
<td>YES</td>
</tr>
<tr>
<td>Fishing nets have been preserved?</td>
</tr>
<tr>
<td>YES</td>
</tr>
<tr>
<td><strong>d. Penetrating wounds</strong></td>
</tr>
<tr>
<td>YES</td>
</tr>
<tr>
<td>Describe gunshot wounds, puncture wounds, from harpoon, parallel deep wounds, etc.</td>
</tr>
<tr>
<td><strong>e. Mutilations</strong></td>
</tr>
<tr>
<td>YES</td>
</tr>
<tr>
<td>Describe cutus, tears, cracks in the shell, missing appendages, etc.</td>
</tr>
<tr>
<td><strong>f. Suspected haemorrhages and haematomas (ventral neck, limbs)</strong></td>
</tr>
<tr>
<td>YES</td>
</tr>
<tr>
<td>Describe extension and area.</td>
</tr>
<tr>
<td><strong>h. Post-mortem damage from scavengers and opportunists</strong></td>
</tr>
<tr>
<td>YES</td>
</tr>
<tr>
<td>Describe extension and area.</td>
</tr>
</tbody>
</table>
### 3. INTERNAL EXAM

<table>
<thead>
<tr>
<th>a. Muscular hemorrhages</th>
<th>YES</th>
<th>NO</th>
<th>ND</th>
<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Describe extension and area.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>b. Fractures</th>
<th>YES</th>
<th>NO</th>
<th>ND</th>
<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Describe.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>c. Content of airway and lung</th>
<th>AIR</th>
<th>FLUID</th>
<th>FOAM</th>
<th>ND</th>
<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Describe lungs’ appearance (heavy, consolidated areas, color variations, etc.) and airway’s content.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>d. Stomach content</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Describe stomach content, amount, presence of parasites and foreign bodies.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stored in frozen</th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>e. Histopathology</th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
</table>
### 3. INTERNAL EXAM

<table>
<thead>
<tr>
<th>f. Presence of macroscopically visible lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>YES</td>
</tr>
<tr>
<td>Describe.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>g. DIAGNOSTIC HYPOTHESIS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
</tbody>
</table>
# ANNEX V

## SAMPLES SCHEMA

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Life History</th>
<th>Genetics</th>
<th>Parasites</th>
<th>Histo.</th>
<th>Contam.</th>
<th>EnteroBT</th>
<th>HerpesVR</th>
<th>Biotox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal</td>
<td>(Frozen or fixed)</td>
<td>(Frozen &amp;/or DMSO)</td>
<td>(70% EtOH)</td>
<td>(10% Formalin)</td>
<td>(Foil wrapped and frozen)</td>
<td>(Culture swab)</td>
<td>(Frozen9)</td>
<td>(Frozen)</td>
</tr>
<tr>
<td>Blood/Serum</td>
<td>(Frozen or fixed)</td>
<td>(Frozen &amp;/or DMSO)</td>
<td>(70% EtOH)</td>
<td>(10% Formalin)</td>
<td>(Foil wrapped and frozen)</td>
<td>(Culture swab)</td>
<td>(Frozen9)</td>
<td>(Frozen)</td>
</tr>
<tr>
<td>Brain</td>
<td>(Frozen or fixed)</td>
<td>(Frozen &amp;/or DMSO)</td>
<td>(70% EtOH)</td>
<td>(10% Formalin)</td>
<td>(Foil wrapped and frozen)</td>
<td>(Culture swab)</td>
<td>(Frozen9)</td>
<td>(Frozen)</td>
</tr>
<tr>
<td>Carapace</td>
<td>(Frozen or fixed)</td>
<td>(Frozen &amp;/or DMSO)</td>
<td>(70% EtOH)</td>
<td>(10% Formalin)</td>
<td>(Foil wrapped and frozen)</td>
<td>(Culture swab)</td>
<td>(Frozen9)</td>
<td>(Frozen)</td>
</tr>
<tr>
<td>Esophagus</td>
<td>(Frozen or fixed)</td>
<td>(Frozen &amp;/or DMSO)</td>
<td>(70% EtOH)</td>
<td>(10% Formalin)</td>
<td>(Foil wrapped and frozen)</td>
<td>(Culture swab)</td>
<td>(Frozen9)</td>
<td>(Frozen)</td>
</tr>
<tr>
<td>Fat deposition</td>
<td>(Frozen or fixed)</td>
<td>(Frozen &amp;/or DMSO)</td>
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STANDARD PROTOCOL FOR POST-MORTEM EXAMINATION ON CETACEANS

Sandro Mazzariol
DVM, PhD

Cinzia Centelleghhe
DVM
Many protocols have been developed for marine mammals necropsy and sample collections, some for taxonomic groups and others aimed to reveal any human interaction. This protocol is a summary of these procedures and our experience providing a practical approach for networks conduction routine data and sample collection. Furthermore, This protocol is designed to improve knowledge about diseases of free-ranging cetaceans by providing guidelines for people to complete more comprehensive necropsies and disease testing.

Using standardize necropsy protocols is important to facilitate comparison of data among stocks or population. Screening for specific pathogens (i.e., Brucella, DMV, and Toxoplasma gondii) is of increasing importance for assessing population health and the presence of potential zoonoses. In some cases, negative results are as meaningful as positive ones. It is our hope that once people on the Adriatic Sea realize the need to learn more about diseases of cetaceans, this protocol also will increase the number of complete postmortem necropsies performed on stranded dolphins.
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1. EXTERNAL EXAMINATION

1.1 Condition code
Before initiating the necropsy, carcass condition must be determined. Preferably, necropsies are performed on fresher carcass (within 48 hours of death); however, environmental conditions can greatly impact condition code. If human interaction is suspected or forensic data are of value, necropsies should be performed irrespective of tissue quality. Carcasses are classified in one of five code categories depending on the level of decomposition.

**Code 1**: Alive or just died (< 2 hours post mortem).

**Code 2**: Fresh carcass (< 24 hours post mortem). Normal appearance, usually with little scavenger damage, fresh smell, minimal drying and wrinkling of skin, eyes and mucous membranes, eyes clear, carcass not bloated, tongue and penis not protruded.

**Code 3**: Moderate decomposition. Carcass intact, bloating evident (tongue and penis protruded) and skin cracked and sloughing, possible scavenger damage, characteristic mild odor, mucous membranes dry, eyes sunken or missing.

**Code 4**: Advanced decomposition. Carcass may be intact, but collapsed, skin sloughing, often severe scavenger damage, strong odor, blubber or muscle easily torn or falling off bones, liquefied internal organs.

**Code 5**: Mummified or skeletal remains. Carcass completely desiccated, often with dried skin draped over bones.

1.2 Nutritional condition
The body condition of a cetacean can be assessed by looking along the dorsal axis of the animal. The dorsal muscle mass (epaxial muscle) to either side of the dorsal fin of a robust animal will be rounded or convex. A thin animal will have a slight loss in epaxial muscle girth and could have a minor sunken aspect to the dorsal-lateral body. An emaciated animal will have a greater loss of epaxial muscle girth and will be concave down the dorsal-lateral body. Emaciated animal may also have more prominent indentation at the nape.
1.3 Sex determination

To determine the sex of a small cetacean, examine the ventral midline of the animal. Both male and female cetaceans possess a genital slit between the umbilicus and anus. For female cetaceans, there should generally be less than 10 cm distance between the centers of the anal opening and the genital slit. Whereas with a male, the distance between the anus and genital slit is much greater. A single short mammary slit can be seen on either side of the genital slit in most female cetaceans, though some males may also possess this feature.

A more definitive method to sex a cetacean is by blunt-probing the genital slit. If the probe angles forward it has entered the vagina and is, thus, a female. If the probe angles backward it has entered the penile opening of a male (often the distal end of the penis can be felt as well). When probing, be sure that your finger has penetrated past the first knuckle in order to ensure accurate sex determination.

It is important to note also, that different species are easier to probe than others; common dolphins are often quite difficult to accurately probe due to very small genital apertures. Final confirmation of gender will always be a result of internal examination.

1.4 Age estimation

Estimation of age for specific animals is important from an epidemiological perspective, as well as important in understanding the basic biological characteristics of a particular species. Currently age is estimated primarily from counts of growth layer deposited in several persistent tissues, primarily teeth and, less often, bone. Saving teeth or other tissue for aging from known-age animals is also important, because these tissue are used to validate the interpretation of growth layers for specific taxa.

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), fusion of epiphyses, pelage color, or reproductive parameters.

Among the physical parameters, length definitely helps to define the estimated age. The average length allows firstly to differentiate whether it is a subject newborn or adult. Few days’ infants can be identified by the presence of lingual papillae and a navel patent. The intermediate length between an adult and a newborn allows to classifying the animal as young. Finally, an old animal is characterized by a size comparable to that of an adult one, associate with aspects of muscle atrophy along the trunk and teeth missing or excessively worn.
1.5 Integument
An external examination should include the investigation and description of the eyes, mouth, blowhole, umbilicus, genital opening and anus.
When examining the eyes, look for discoloration, injuries, or discharge. Document any lesions, parasites, and the mucus membrane color in the mouth. Make note of worn, broken or missing teeth.
Describe color and amount of discharge from blowhole as well as the presence of parasites or obstructions. Obtain culture swabs.
Examine the umbilicus in neonates for signs of infection and degree of healing. Look for lesions, discharge or growths around the genital opening and anus. Obtain samples of abnormalities for histology, microbiology and molecular investigations.
If the animal has mammary glands, attempt to express milk and note color, consistency and estimate amount in cc’s or mls. Milk can be expressed by pressing on the body about 10cm dorsal and cranial to the mammary slit and massaging downward toward the slit.

1.6 Teeth
Teeth from the center of the lower left mandible are collected for life history analysis. Using a scalpel blade, transversely cut in between and around 5 -7 teeth. Teeth can be extracted by inserting tooth extractor or a flat head screwdriver in the incision made between the teeth and wiggling the tool down to the base of the mandible until the entire, undamaged, tooth becomes loose. Avoid snapping or crushing the tooth, as such damage can render the sample useless for analysis. In some species and in older animals, a sturdy knife may be advisable over a scalpel to avoid breaking the blade.

<table>
<thead>
<tr>
<th>Species</th>
<th>Tot. length birth (cm)</th>
<th>Tot. length pup (cm)</th>
<th>Tot. length 1 year (cm)</th>
<th>Tot. length 2 years (cm)</th>
<th>Tot. length weaning (cm)</th>
<th>Tot. length adult (m)</th>
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<td>170-225</td>
<td>225</td>
<td>2,2-3 cost. 2,5-6 pel.</td>
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Estimated age based on animal’s length
1.7 Skin and Blubber
Examine and document any scars, abscesses, ulcerations, erosions, wounds and parasites on the skin. Make note of the size (length x width x depth/height), shape, color, texture, location and distribution of all abnormalities.
Remove about two cm² of skin from the tip of the dorsal fin or flukes for genetic (frozen and DMSO) and histology samples. A skin sample with no blubber attached is preferred. Trim the skin as cleanly as possible from the other tissue. (Be sure all morphometric measurements have been completed before collecting this sample.)
The first step to examine the body cavity of the animal is removal of the blubber. Position the animal left side up. Using a scalpel blade or knife, start just left of the dorsal midline posterior to the blowhole and make a longitudinal incision down the length of the animal ending at the dorsal tail stock. Do not penetrate into the skeletal muscle, cut only through the skin and blubber layers. Next, make a dorso-ventral incision perpendicular to the previous body length incision just cranial to the anterior insertion of the left pectoral flipper. Continue making perpendicular incisions down the length of the animal that are ~20 cm apart, creating a series of panels along the lateral body. At the top of each panel begin to separate the blubber from the muscle by cutting through the fascia or connective tissue. If you remain between the blubber/muscle interface (fascia) and reflect the panel of skin down and away from the body, in a dorsal to ventral direction, the blubber should easily separate from the muscle.

Note the thickness, color and texture of the blubber. Look for parasites and abnormalities within the blubber layer.

Obtain blubber samples for histology and contaminants. When collecting these samples, be sure to collect blubber without any skin or muscle attached and be sure to take the sample from the same location on each carcass, generally from the dorsal mid-thoracic region.

Once the blubber has been examined, make a cut along each reflected panel at the ventral midline and discard the blubber.
1.8 Skeletal Muscle
Examine the quality of the fascia and muscle on the body before removing it. Note the color, texture, thickness and abnormalities. Look for hemorrhage, post mortem pooling of blood in vessels (hypostasis or post mortem lividity) and bruising (hematoma). Bruising usually has a gelatinous texture and is deep maroon to purple.
Remove the large dorsolateral muscle mass or epaxial muscle that spans from the occipital ridge down to the tail stock. Use the dorsal and lateral spinal processes as landmark boundaries for this muscle. Trim away as much muscle as possible from the backbone and ribs.
Obtain muscle samples for histology and contaminants.
2. INTERNAL EXAMINATION

2.1 Removal of the Scapula and Prescapular Lymph Node
Locate the prescapular lymph node prior to the complete removal of the scapula. The oval to triangular shaped, beige to peach tissue is located just underneath the cranial corner of the scapula, proximal to the location of the external ear.
Normal lymph nodes throughout the body usually share the same characteristics: a well-defined oval shape, slightly firm texture, color is diffusely beige to peach, with very slight differentiation between the cortex (outer layer) and medulla (center area). If the tissue begins to vary from the homogenous peach to tan it is indicative of a reaction.
Note the size, shape, color and texture of the prescapular lymph node. Be sure to distinguish changes of the cortex from changes of the medulla. Sample for histology, microbiology and molecular investigations.
Remove the left scapula and appendage by cutting through the connective tissue and muscle just underneath the bone. If you pull the scapula ventro-laterally, reflecting it down as done with the blubber layer, the scapula will detach easily. You should hear a crackling sound as you pull and cut indicating that you are in the correct spot between muscle groups.

2.2 Removal of the Rib Cage
Before collecting any samples or cutting the ribs, the diaphragm should be punctured with a scalpel or scissors and deflation should be noted. If the diaphragm is already deflated, it is possible that a pneumothorax or severe pneumonia may be present.
To open the thoracic cavity, start at the caudal end of the left rib cage and feel for the articulation between each individual rib and vertebrae. The ribs and vertebrae should easily separate, without breaking, if you cut through the articulation with a scalpel blade or small knife. Also to note, age and disease may affect the way the joints disarticulate. Move cranially from rib to rib maintaining a constant angle with your scalpel as you cut and moving the rib to find the articulation.
Note that the most cranial ribs are double-headed along the vertebrae. The doubleheaded ribs can be removed by first severing the first articulation, then by sliding the scalpel along the inside of the second head to reach its articulation with the spine. The articulation can be cut by sweeping the scalpel parallel to the long axis of the animal. The rib articulations should feel smooth, not granular. Feel for fractures and bone spurs on the rib cage.
Removed in this manner, the skeleton may be of more value for future bone pathology studies, educational outreach or as museum specimens.
Once the rib cage is removed, examine the body cavity with all organs in place. Note any discoloration, lesions, adhesions, odor or fluids.
At this point, one needs to adhere to a systematic examination of the internal tissues. The organs may be removed as a pluck, or may be examined in situ. The method of sampling can be guided by sampling needs, condition code, and personal preference. It is recommended that internal fluids, such as those in the gastro-intestinal system do not contaminate other tissues.
2.3 Thyroid
The thyroid sits ventrally on the cranial trachea and spans the width of the trachea. The thyroid is one of the more difficult tissues to locate and identify. The color and texture are often similar to smooth muscle. The parathyroid is a small, light colored tissue attached to the thyroid along the cranial margin of the thyroid and can aid in correct tissue identification if it can be found. Examine the tissue externally and internally.
Note the size, shape, color and texture. Sample for histology, microbiology and molecular investigations.

Thoracic cavity (Stenella coeruleoalba)
2.4 Thymus
The thymus is a large, lymphoid organ, that is primarily found in neonates and some juveniles. It is situated at the base of the thoracic inlet, cranial to the anterior margin of the heart. The thymus is absorbed with time after weaning, thus is not usually visible in adult marine mammals. Examine the tissue externally and internally. Note the size, shape, color and texture. Sample for histology, microbiology and molecular investigations.

THORACIC CAVITY

2.5 Tracheobronchial Lymph Node
The tracheobronchial lymph node is located along the distal cranial ventral surface of the lung proximal to the bifurcation of the trachea. It can easily be located by reflecting the cranial lung tissue away from the cavity and palpating the connective tissue between the lung and anterior to the trachea bifurcation. It is recommended that this tissue be identified and removed prior to removal of the lung or trachea, as it can be easily lost without anatomical landmarks. Examine the lymph node externally and internally. Describe any differences between the cortex and medulla. Note any other changes in size, shape, color and texture. Sample for histology, microbiology and molecular investigations.

2.6 Esophagus
Trace the esophagus from the exposed caudal end to the mouth, opening the esophagus in the same manner as done with the trachea. Observe the serosal and mucosal surfaces of the esophagus. Note, color, texture and contents. Sample for histology.
2.7 Trachea
The trachea is a long, firm, off-white, flexible, ridged, tubular organ that extends from the larynx to the tracheal bifurcation. Using scissors, cut through the entire length of the trachea from the bifurcation up to the apex of the thorax. Examine the mucosa and identify and describe contents (froth, fluid, blood, color, etc.). Sample for histology.

2.8 Lungs
The lungs occupy the majority of the thoracic cavity and are the large, normally bright pink, tissue with a consistent sponge-like texture. Detach the lung from the trachea at the bifurcation. Examine the pleural surface: note color pattern and texture. Normal, air-filled lung tissue should bounce back immediately after being depressed with a finger (like a sponge) and float when placed in water or formalin. To examine the internal structures, using scissors, trace the trachea from the bifurcation along the bronchi and into the bronchioles of each lung. Note whether fluid, froth and/or parasites are present and describe amount, color, etc.

Next, make serial cuts into the tissue by “bread-slicing” (making multiple, parallel slices into the tissue) perpendicular to the long axis of the body to examine the parenchyma. This is best done with a long knife using a single sweeping cut in order to avoid tearing or serrating the lung tissue. Examine the parenchyma and note color pattern and texture. Sample for histology, microbiology and molecular investigations.

Lung (Tursiops truncatus)
2.9 Heart Muscle and Valves

Before handling the heart, observe and describe the pericardium. There should be a small amount of clear fluid within the pericardium to allow for lubrication. Note if there is excessive fluid and describe the characteristics. Also, note the presence of gas bubbles within the pericardium and vessels and note thickness of the tissue.

Trim away the pericardium and observe the epicardium (external surface of heart) in situ. Note size, color, and texture of each structure (right and left atria and ventricles, aorta, and pulmonary vessel).

Remove the heart by cutting transversely across the aorta and pulmonary artery leaving approximately 6.0 cm of each vessel still attached to the heart muscle. There are varying techniques for examining the internal structures of the heart. One way is to use scissors to make a small opening in the cranial right atrium and cut down along the medial edge of the right ventricle down to the apex. Continue cutting along the right ventricle side of the septum until this chamber joins the pulmonary artery and cut up through the vessel. Next, snip the left ventricle side of the apex, cut through the muscle along the septum, and up through the aorta. This process leaves both sides of the heart intact.

A simpler way to examine the endocardium (inner surface of the heart) is by slicing the organ completely in half starting at the apex going laterally toward the vessels, so that it opens up like a book. Examine each chamber for the presence of worms or other foreign matter. Note the size/thickness of each atrium and ventricle, as well as color and texture. The left ventricle should be substantially thicker than the right.

Thoroughly examine the interior of the valves for changes in texture or thickness. Normal mitral and tricuspid valves should be thin and slightly opaque. Once the endocardium is examined, bread–slice the ventricles to examine changes in the myocardium.

Sample the right and left ventricles and atria, septum, apex, atria and aorta for histology.
2.10 Diaphragm

The diaphragm is the thin, smooth textured, dark maroon, expandable muscle that is attached to the caudal rib cage and separates the thoracic and abdominal cavities. Note the texture and color as well as any tears or adhesions. White striations over the surface of the diaphragm are normal. Trim away the diaphragm enough so that there is complete access to the abdominal organs. Sample for histology.

ABDOMINAL CAVITY

To expose the abdominal organs, incise the abdominal wall from the last rib mid-ventral to the level of the anus. Extend the most cranial cut laterally along the thoracic arch and reflect the abdominal musculature to expose the internal tissues. The orientation of the organs should be visually assessed and any free fluids aseptically collect in a sterile syringe prior to proceeding with the internal examination.

2.11 Liver

The multi-lobular, diffusely maroon liver is large, lies over the stomach and dominates most of the abdominal cavity. Examine the parietal (toward the body wall) and visceral (toward the organs) surfaces of the liver and note color pattern, texture and size of the lobes. Examine the parenchyma of the liver by bread-slicing through the tissue. Again, note the color and texture within. Examine bile ducts for presence of parasites. Sample for contaminants, histology, microbiology and molecular investigations. All cetaceans lack a gall bladder.

Liver (Tursiops truncatus)
2.12 Spleen
The shape and size of the spleen vary among cetacean species. The spleens of most dolphin are palm-sized, spherical and mottled dark purple to white with a smooth external texture. For other species, the spleen may share these characteristics or be smaller and oblong. Regardless of physical characteristics, the organ is always located underneath the main stomach toward the left side of the body. Remove the spleen by detaching it from the omentum (thin, web-like, connective tissue). Note size, shape, color and texture of both the surface and the parenchyma of the spleen. In some cases, smaller (0.2—1.0cm), accessory spleens may be attached to the visceral surface of the spleen. These smaller spleens share the same characteristics as the larger spleen. Sample for histology, microbiology and molecular investigations.

2.13 Pancreas
The pancreas is a peach colored, irregularly shaped, pyramidal, softer tissue that is attached to the mesentery and sits in the curve of the duodenum. Remove the pancreas from the cavity by detaching it from the connective tissue and duodenum. Note the size, shape, color, and texture of the surface. Cut into the parenchyma and note changes in color or texture. Examine ducts for parasites. Sample for histology, microbiology and molecular investigations.

2.14 Mesentery and the mesenteric Lymph Node
The mesentery is a broad sheet of connective tissue which attaches the intestines (and other viscera) to the mesenteric root. This connective tissue should be translucent and show some resistance when attempting to bluntly dissect. Examine the mesentery for parasitic or fungal attachments or other abnormalities. Note thickness and opacity. The mesenteric lymph node is a finger-like, gray to tan colored, larger lymph node that is centrally attached to the mesentery. Remove the lymph node by detaching it from the mesentery. Note the size, shape, and color of the mesenteric lymph node. Examine the external surface and internal structures for changes in color and texture. Unlike previous lymph nodes discussed, the mesenteric lymph node tends to have a more defined cortex and medulla. Be sure to describe each structure separately. Sample for histology, microbiology and molecular investigations.
2.15 Stomach
The stomach of most odontocetes is comprised of three compartments: the fore stomach, the main stomach, and the pyloric stomach. There is also the omentum, net-like connective tissue that is attached to the visceral side of the stomach. To avoid contaminating the remaining tissues in the body cavity or losing contents, it is necessary to tie off both ends of the stomach prior to extracting. With some twine, tie a tight, secure knot at the location of the attachment of the esophagus to the fore stomach. A second piece of twine can be tied just below the base of the pyloric stomach where the small intestines begin. Remove the stomach from the carcass by cutting beyond both knots. Examine the serosal (external) surface of the stomach for discoloration and lesions. If an internal pathology is present, the peri-gastric lymph nodes attached to the stomach should be noticeably enlarged. Sample for histology, microbiology and molecular investigations and make note on the sample inventory list if this is the case.

Otherwise, remove all excess attached tissue from the exterior of the stomach and weigh the stomach full. Using a scalpel, make an incision through the wall along the greater curvature of each stomach large enough to allow examination of the contents and the entire mucosal surface. Describing the contents of each compartment separately, note the composition of stomach contents (fluid; whole or partially digested fish; fish bones; parasites; foreign objects). Be sure to describe amounts, color and texture. Prior to further manipulation, collect a sample of contents for biotoxins. The remaining contents can be emptied and rinsed into a sieve to ensure solid material is not lost and is thoroughly examined. Save all foreign objects for human interaction documentation. Once empty, examine the mucosa of the stomach. Note the color and texture of the mucosa of each compartment separately. The mucosa of the fore stomach is composed of squamous tissue and is usually white. The wall of the main stomach is stratified and usually thicker than that of the fore stomach. The mucosa is usually dark red. The pyloric stomach tends to be thin walled, glandular, and the mucosa is pink or stained with bile. Look for ulcers, areas of discoloration and other abnormalities. Weigh the stomach empty.
Sample each compartment for histology.

Stomach (Tursiops truncatus)
2.16 Small intestine
Examination of the intestines is preferably left until the end of the necropsy so as to not contaminate the other organs. Examine the serosal surface of the small intestine first. Look for areas of hemorrhage or discoloration as well as parasites. The inside of the small intestine can be examined by spot checking: at 5 – 10 random, separate areas, using scissors to cut about 10 cm down the length of the lumen. Note color, consistency and amount of contents as well as thickness of the lumen and the texture and color of the mucosa.
Sample several sections for histology.

2.17 Large intestine
To locate the beginning of the large intestine, look for the ileo-cecum-colic junction, which usually is a ridged junction between the smaller diameter small intestine and the larger diameter large intestine. The large intestine can be examined in the same manner as the small intestine. Note any discoloration or the presence of parasites. Describe the color, consistency and amounts of contents. Note the thickness of the lumen as well as texture and color of the mucosa.
Sample for histology.

2.18 Colon
Examine the serosal surface of the colon for areas of discoloration. Cut through the lumen of the colon from the anus to the large intestine. Describe the color, consistency and amount of contents. Note the thickness of the lumen as well as texture and color of the mucosa. Sample for histology.
Collect feces for biotoxins analysis.

2.19 Adrenal Gland
The right and left adrenal glands are located just anterior to the cranial pole of each kidney and are attached to the dorsal abdominal wall. The adrenal glands are small, oblong, light maroon tissues possessing irregular furrows over the surface. Locating and extracting the adrenals prior to removing the kidneys is highly recommended, as they can be difficult to locate without the kidneys as an anatomical reference.
To remove the adrenals, grasp and pull the tissue away from the body wall and cut the surrounding connective tissue. Before sectioning, measure (LxWxH) and weigh each adrenal. When cut in half, a normal adrenal will present a distinct darkened center (medulla) with a lighter perimeter (cortex).
Note size, shape, color and texture of the external and internal tissue. Also, note relative size of the aperture, or opening in the medulla, which would indicate usage of the vessel. Normal apertures should be no larger than the tip of a pin. Sample each adrenal for histology investigations.
The left and right kidneys are maroon, ovoid, tissues comprised of numerous, clustered *reniculi* (*miniature kidneys*) and are attached to the caudal dorsal abdominal wall. Examine the capsule (connective tissue surrounding the kidney) for the presence of fluid, hemorrhage or bubbles and note color, thickness and opacity. Create a longitudinal incision through the capsule and reflect the margins to assess for adhesions or sub-capsular hemorrhage. Detach the kidney from the abdominal wall and remove the capsule to examine the external surface.

Note the size, shape, external color and texture of each kidney. Examine the internal structure of each kidney by bread-slicing. Note color and presence of stones. Observe the degree of differentiation between the cortex and medulla as well as the medulla/cortex ratio within each *reniculus*. Each *reniculus* should be well demarcated but clustered together within the kidney itself.

Sample for contaminants, histology, microbiology and molecular investigations.
2.21 Urinary Bladder

The bladder is a smaller, light pink, organ that is found along the ventral body wall. The organ may appear as a thick walled, muscular organ, but if distended with urine, the walls may be thinner and semi-translucent.

Before removing the bladder from the body, extract contents using a sterile syringe and medium gauge needle. If none are available, be sure to clamp the bladder using a hemostat before removing the organ in order to retain urine. Note color, consistency, and amount of urine.

Remove the bladder and examine internally by cutting along the length of the organ to expose the mucosal surface. Note color and texture of the mucosa.

Sample the cranial tip of the bladder for histology.

2.22 Reproductive tract

2.22.1 Female: Ovaries and Uterus

The uterus and ovaries can most easily be identified by following the reproductive tract from the vagina to the uterus where it bifurcates to a right and left horn, each ending at the attachment of the ovaries. The uterus is a tan to pink tissue that will vary in size and thickness depending on the maturity of the animal and its reproductive history. Note size, shape, color and texture of the external and internal surfaces of the organ.

If a fetus is present and is too small for a sufficient individual necropsy, incise the abdomen, collect microbiology and molecular samples, then preserve fetus whole in formalin. If the lung tissue floats in formalin (or water), this signifies that bronchiole expansion of the fetal lungs has occurred.

An off-white spindle-shaped ovary is attached to the end of each uterine horn. Detach the organ from the uterus and examine the external surface. Note size, shape, color and texture.

A mature ovary will possess random darkened notches or scars (corpus albicans) which signify previous ovulations. The ovary of a pregnant female will possess a corpus luteum or a large yellow mass attached to the ovary. Before internal examination, measure (LxWxH) and weigh each ovary. Also count and note the number of scars and presence/absence of a corpus luteum. Examine the tissue internally and note color and texture.

Sample both the uterus and ovaries for life history, histology, microbiology and molecular investigations.

2.22.2 Male: Testis and Penis

The elongated, spindle shaped, off-white paired testes are located within the caudal abdominal cavity along the ventral wall, posterior to the kidneys, each one just off the ventral midline. Remove the testes (with the epididymus attached) from the body. Obtain measurements (LxWxH) and weight of each one. Examine the size, shape, color and texture externally and internally. Section epididymus for the presence/absence or sperm. Obtain samples of each testis for life history, histology, microbiology, molecular and ancillary investigations.

Examine the penis externally and look for discharge or the presence of papillomas or other lesions.
SKULL

2.23 Removal of the Brain

The brain is the most fragile and easily disrupted tissue in the entire body, thus extreme care must be taken when removing the brain from the skull. Before removing the head, cerebrospinal fluid (CSF) can be collected for cytology and culture. To do so, remove the overlying soft tissue at the back of the head and neck to gain access to the atlanto-occipital joint. Insert a sterile needle and syringe and collect the clear, viscous CSF.

First the head must be detached from the body to safely remove the brain. Do so by cutting behind the blowhole down to the joint between the skull and cervical vertebrae, and then completing the cut ventrally. Once separated, remove all excess skin, blubber, muscle and connective tissue from around the dorsal and caudal skull. Then, using a Stryker saw or a hacksaw, make cuts from left to right through the middle of each occipital condyle, up each side of the lateral skull, and then across the dorsum, just posterior to the marked transverse ridge at the apex of the skull. Be sure to fully penetrate the bone, but avoid contact with the brain. This can be very difficult, so proceed with caution. It will take some practice to successfully remove the cranium without penetrating the brain. Carefully place a chisel between the cut bone and turn the tool to crack the remaining bone until the back of the skull comes away in one piece. Be careful to pull it off evenly, without using one edge as a lever, otherwise the bony shelf (the tentorium cerebellae) that is positioned between parts of the brain will penetrate the tissue and damage the brain. Using fingers, gently tease the meninges (thin membranes enveloping the brain) away from the skull, and work under the brain to sever each cranial nerve. Inversion of the head often allows the brain to gently descend in to the palm of your hand.
2.24 Examination of the Brain

Again, the brain is the most delicate tissue in the body and will fall apart if handled excessively. Observe the external surface of the brain and note symmetry of each distinct structure (right and left cerebral hemispheres, cerebellum, and brain stem) while noting the color, texture and presence of worms or lesions. Vascular congestion can be a result of positioning or post mortem lividity.

Cut through the brain in one long motion, cranial to caudal, using a large, thin knife so that the two hemispheres evenly separate. Again, note symmetry, color, texture and the presence of worms or lesions. Each section of the brain has a distinct pattern. The cerebrum is comprised of two distinct lobes and is the most cranial section of the brain. The cerebellum is the most caudal portion and sits dorsal to the brain stem. The brain stem originates from the ventral midline of the brain and extends into the spinal cord. Sample the cerebrum, cerebellum and spinal cord for microbiology and molecular investigations. Fix the remaining brain tissue for histology. It is important to include a sample of normal and abnormal meninges in the histology sample set.
2.25 Pituitary Gland
Once the brain has been removed, immediately under the crossover of the optic nerve, the usually small pituitary gland can be extracted after incision through the overlying dura. The organ is within a bony recess and has to be lifted out using a scalpel blade and small forceps. Sample for histology and other priority testing.
3. SOUND EXPOSURE

The possibility that exposure to sonar stimuli could lead to the stranding of cetaceans is an issue of increasing interest because of recent strandings temporally and geographically coincident with naval sonar operations. Nonauditory link between strandings and sonar exposure is proposed to occur when tissues are supersaturated with dissolved nitrogen gas, and bubble growth–facilitated diffusion is stimulated within tissues. Bubble growth could result in emboli-induced tissue separation and increased localized pressure in tissues, the presumed cause of decompression sickness (DCS) in human divers. DCS is the result of the supersaturation of body tissues with nitrogen gas and the subsequent release of nitrogen gas bubbles. No studies have specifically addressed whether nitrogen bubble formation in tissue occurs in diving marine mammals. Recently researchers have presented strong evidence of chronic, gas bubble lesions in the liver and kidney. These lesions of different stranded cetacean species suggest that gas bubbles formed in vivo can persist and generate fibrosis in diving cetaceans.

Hemorrhage in the brain, ears and acoustic fat was reported as the main lesions.

3.1 Fat emboli

The presence of gas emboli is an important finding in human DCS, but pulmonary fat emboli have also been reported with DCS-related, severe cardiorespiratory disturbances. Systemic fat embolism is a secondary effect of the abrupt pressure changes observed with dysbaric osteonecrosis (DON), a condition initiated by the evolution of gas bubbles in nitrogen-supersaturated fatty marrow after inadequate decompression. The clinical manifestations of fat emboli depend on the volume of fat reaching the lungs and other affected tissues.

The pathogenesis of fat embolism is not fully understood, and it is likely multifactorial. Two mechanisms have been proposed for the development of fat emboli. First, direct entry of fat emboli into the bloodstream after trauma may cause direct, toxic injury in the lung and produce respiratory insufficiency when free fatty acids are released from fat tissues. A second mechanism involves the generation of fat emboli from plasma lipoprotein disruption and coalescence of lipid at the intravascular gas bubble interface.

In particular, hemorrhages should be detected and reported in acoustic fat in lower joint, around temporomandibular joint, around middle and inner ear, in the sub-cutaneous tissue, in inner cavities, around meningeal tissue or around eyes.

Tissues should be collected and stored in buffered 10% formalin and later fixed and preserved for routine histopathologic examination or immediately frozen. Tissues from stranded animals include: brain, cervical spinal cord, lung, liver, kidney, heart, middle and inner ear.

Histologic stains used on frozen or on formalin-fixed tissues to detect fat emboli are oil red O stain (ORO) and Sudan black B stain. Furthermore pulmonary tissue and periotic veins could be processing according to Osmium tetroxide post-fixation technique in order to detect fat emboli within vessels.

3.2 Gas-bubble

Gas-bubble lesions have been described in cetaceans stranded in spatio-temporal concordance with military maneuvers. Authors described an acute and systemic gas and fat embolic syndrome similar to decompression sickness (DCS) in human divers. A behaviorally induced decompression sickness-like disease was proposed as a plausible causal mechanism, although these findings remain scientifically controversial. Investigation into the constituents of the gas bubbles in suspected gas embolic cases are highly desirable.

Gas chromatography has been demonstrated as a valid method to discriminate putrefaction gases from air embolism and has been used as a forensic tool in humans for this purpose.

Although gas extraction can sometimes be performed at the stranding site, gas analysis must always take place in a laboratory, a situation that requires proper storage and transportation of gas samples. Gases
likely to be of scientific interest might be found in sites within body cavities (such as intestinal gas, air in sinuses, pneumothorax, subcapsular gas or gas pockets), inside vessels (emboli) and/or mixed with blood inside the heart. Sampling of gas from these body sites will require the use of different techniques.

A standard necropsy protocol is used, with some modifications necessary to preserve bubbles for gas collection and storage. Skin and blubber are removed taking into account possible gas embolism within the subcutaneous veins. If bubbles are seen, vessels are explored to confirm that they have not been cut during dissection. As soon as a bubble is detected, a photograph is taken and the bubble is sampled for gas analysis. In order to have access to the body cavities, dissection is done avoiding cutting of medium-large vessels (usually larger than 3 mm, although it varies among species). The abdominal cavity is opened first and mesenteric and renal veins as well as the lombo-caudal venous plexus are screened for bubbles. The thoracic cavity is then opened to permit access to the heart and coronary veins are explored. After bubble exploration and gas sampling from the different localizations, systemic vessels can be subsequently cut and the routine necropsy protocol can be completed.

When sampling gas from sites within body cavities, the 5-mL additive-free vacutainer is directly applied to cavities with its appropriate plastic holder or adapter and a double-pointed needle with a rubber barrier on the tube puncture side. To avoid atmospheric air, the needle is preliminarily inserted into the cavity for purging; the vacutainer is then pushed against the double-pointed needle and, finally, the vacutainer is removed before the needle is released from the cavity. This technique allows adequate gas sampling from head sinuses, the digestive tract and even from heart chambers if post mortem autolysis ranged from grade 3 to grade 5. The pericardial sac is always filled with distilled water to avoid contamination with atmospheric air.

If the carcass preservation status is fresh or very fresh (putrefaction grades 2 or 1, respectively), the aspirometer is used to separate the gas from the blood found in the heart.

In order to properly sample bubbles in the rest of the cardiovascular system (blood vessels), disposable insulin syringes (BD Plastipak U-100 insulin) are used to sample bubbles and their contents are promptly injected into a vacutainer. One new syringe and one new vacutainer are used for each bubble.

![Image](image_url)

a. The new design of the aspirometer – b. close-up view of intravascular bubbles in the coronary heart veins of a beaked whale – c. abdominal cavity with close-up view of the spleen showing chronic gas embolism. SCIENTIFIC REPORTS | 1 : 193 | DOI: 10.1038/srep00193

[Image of equipment and tissue samples]
3.3 Ear

Ear sampling could be used in both in case of sonar exposure or other sounds exposure. Initially, to collect the ear, disjoint the jaw to expose the tympanic bulla and clean it from the soft tissues. Helping levering with a scalpel or knife, cut the ligament between the tympanic complex, the occipital and temporal bones up to completely remove the whole bone. Once removed, locate the eardrum and slowly inject a solution of 10% Formalin (better for fall).

To evaluate the micro and macroscopic examination of the inner ear, it’s necessary to decalcify the bones using a fat commercial decalcifier (RDO®), based on hydrochloric acid, according to the protocol proposed by Morell et al. (2009). Times in RDO® must be adapted to different species and size of the bone. Decalcification finished, proceed to carry serial section of the ear itself.

In larger species should remove the tympanic complex to facilitate the decalcification of the periotic process.
4. SAMPLING PROTOCOL

4.1 Life History
Code 1, 2 and 3, ideal; 4 and 5 limited.

- **Age determination**
  - Teeth, should be kept frozen.
  - Eyes, should be kept frozen.
  - Ear plugs, preserve in 10% NBF.

- **Reproductive Status**
  - Gonads and Uterine samples fixed in 10% NBF.

- **Feeding Habits**
  - Stomach contents can be collected into a sealable plastic bag or jar, freeze.
  - Skin, for stable isotopes should be kept frozen. What the animal has been eating recently.
  - Baleen, provides a multiple year record of the animal’s diet.

4.2 Genetics
Codes 1, 2-4 are suitable. Only one of the following needs to be collected, unless there are specific requests.

- **Skin, Heart, Liver** - Better to freeze tissue samples, in case the tissue is used for something other than genetics. Genetic tissue samples can be fixed in DMSO saturated with NaCl.
- **Blood** - Can only be collected from Code 1 and 2 animals. Minimum amount is ~10 ml; 50-100 ml is optimal for DNA studies.

4.3 Microbiology
Code 1 and 2 are ideal, 3 limited, 4 and 5 useless. Take separate samples for bacteriology and virology. Lesions should be sampled from several distinct locations, include normal tissue with the infected tissue sample.

- **Bacteriology** – Avoid freezing samples for bacteriology if avoidable. Refrigerate samples at 4°C. Freezing at –70°C is preferable to decomposition.
  - External samples can be taken with a swab from the eye, blowhole, throat, anus, and genital opening. Culture swab in a bacterial transport medium.
  - Internal samples can be taken from the heart, lungs, liver, spleen, lymph node, bone with marrow, and tissues showing pathological changes. 6 x 6cm sample placed in a sterile container.
  - Fluid samples can be taken from the pleural fluid, peritoneal fluid, urine, blood, fluid from abscesses. Store in appropriate aerobic or anaerobic vial.

- **Virology** – Refrigerate samples at 4°C.
  - External samples use a sterile swab dipped in viral transport medium. Take samples from the blowhole, rectum, genital opening. Place swabs in the vial that contains the viral transport medium.
  - Internal samples can be taken from the CNS tissues, lungs, liver, spleen, kidney, placental/fetal tissues, tissues with pathological changes, intestinal contents. 6 x 6cm sample placed in a sterile container.
  - Fluid samples from pleural fluid, peritoneal fluid, pericardial fluid, urine, fluid from skin vesicles, blood from heart, cerebrospinal fluid.
4.4 Parasites
Samples taken from Code 1-4 animals are suitable for examination.
- **Barnacles** – first fix in 10% NBF, for no more than 24 hrs, then transfer to 70 % EtOH.
- **Copepods & Amphipods** – place directly into 70% EtOH.
- **Nematodes (roundworms)** – Fix in GAA for 5-10 minutes first if possible. Otherwise use 70% EtOH or 10 % NBF. If formalin is used, fix only for a few hrs. Then transfer to 70% glycerin alcohol.
- **Trematodes (flukes/flatworms)** – Dead or alive, fix in AFA for up to 3 days, transfer to 70% EtOH. Do Not use glycerin alcohol.
- **Cestodes (tapeworms)** – Fix for 5-10 min. in AFA solution and water, 4:1 ratio. Transfer to 70% EtOH. Include cestode head when removing from the host, if necessary cut host tissue.
- **Acanthocephalans** – Fix in AFA for up to 24 hrs. Then transfer to 70% glycerin alcohol.

4.5 Histopathology
Code 1, 2 and 3 are ideal. Rare / Endangered species should be thoroughly sampled. Lesions, fractures, lacerations, and gunshot wounds of any code should be sampled in this manner.
- Tissues should be preserved in 10% NBF.
- Tissue samples should be no larger than 3 x 3 cm and approximately 1 cm in thickness.
- Ideally, histo samples should be cassetted and placed into a labeled jar for the appropriate Institution/Researcher. Individual requests should be noted and tracked.
- Samples of gross lesions should include abnormal and normal tissue.

4.6 Contaminants / Biotoxins
*(organochlorines, heavy metals)*
Code 2 is ideal, 1 and 3 is limited; 4 and 5 questionable to useless.
- **Biotoxin Analysis** – Code 2 animals only. Collect stomach contents, liver and/or kidney tissue. Freeze.
- **Contaminant Analysis** – All of the samples should be frozen in plastic zip lock bags.
  - Blubber (include skin), Muscle, Liver, Kidney, Brain (if possible include cerebrum and cerebellum).
5. BIBLIOGRAPHY


6. NECROPSY REPORT

<table>
<thead>
<tr>
<th>Event Info</th>
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<tbody>
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CARCASS DISPOSITION:

HISTORY:

COMMENTS:

Necropsy Observations: Please note general observations of color, condition, textures, etc. even when utilizing NA= not applicable, NE= not examined, NSF= no significant findings, NVL= no visible lesions. List weights (g) next to each organ examined.
**EXTERNAL EXAM**


**Skin / Hair Coat (color, condition):**

Wounds / Scars:

Lesions:

Parasites:

Nostrils / Blowhole:

Mouth (tongue, teeth condition, ulcers) / Mucous membranes (color):

Eyes (discharge, color, ruptures):

Ears:

Genital slit / anus:

**Umbilicus:** Pink Open Healed:
**EXTERNAL EXAM**

**MUSCOLO/SKELETAL SYSTEM**

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**CIRCULATORY SYSTEM**

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**PULMONARY SYSTEM**

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GASTROINTESTINAL SYSTEM

Esophagus:

Stomach (contents, ulcers, mucosa, parasites):
Weight Full: _______  Weight Empty: _______

Small Intestine:

Large Intestine:

Colon:

Omentum, Mesentery, Peritoneum:

Liver (color, congestion, lesions, size):

Gall Bladder / Bile Duct / Pancreaticoduodenal Duct (color, amount):

Pancreas:

URINARY/REPRODUCTIVE SYSTEMS

Kidneys (reniculi differentiation, color, condition):
(R)

(L)

Bladder:
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<td>(L)</td>
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</table>

Mammary glands:

Uterus / Cervix / Vagina:

Pregnant? : [Y] [N] [NA (male)]

LYMPHATIC SYSTEM

Thymus:

Spleen:

Scapular Lymph Node:

Tracheobronchial Lymph Node:

Mesenteric Lymph Node:

Other Lymph (list location):
## ENDOCRINE SYSTEM

### Thyroid:

### Adrenals:
- (R) \( L \times W \times H \) cm:
- (L) \( L \times W \times H \) cm:

### Other:

## CNS

### Spinal Cord:

### Brain:

### Pterygoid Sinuses:

## OTHER

### Thoracic Cavity:

### Abdominal Cavity:

### Internal Parasites (location, type, number):

## Differential Diagnosis from Gross Exam:
## 7. PROTOCOL FOR EXAMINING CETACEANS FOR SIGNS OF HUMAN INTERACTION

### 1. GENERAL INFORMATION

<table>
<thead>
<tr>
<th>N. ID</th>
<th>Species</th>
</tr>
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<tbody>
<tr>
<td>Sex</td>
<td>Length</td>
</tr>
<tr>
<td>Cause of death</td>
<td>Date of death</td>
</tr>
<tr>
<td>Location of necropsy examination</td>
<td>Date of exam</td>
</tr>
<tr>
<td>Video</td>
<td>YES</td>
</tr>
<tr>
<td>Photo</td>
<td>YES</td>
</tr>
<tr>
<td>Conservation Code</td>
<td>Fresh</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Note</td>
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</tr>
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</table>

**ND**: Not Determined – **NE**: Not Evaluable

### 2. EXTERNAL EXAM

#### a. Body condition

<table>
<thead>
<tr>
<th>Emaciated</th>
<th>Not emaciated</th>
<th>ND</th>
<th>NE</th>
</tr>
</thead>
</table>

#### b. Signs of fishing net or lines.
(indicate if YES, NO, ND, NV for each area and in the positive case describe the lesion)

<table>
<thead>
<tr>
<th>Head</th>
<th>Dorsal fin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pectoral fin left</td>
<td>Pectoral fin right</td>
</tr>
</tbody>
</table>
### 2. EXTERNAL EXAM

<table>
<thead>
<tr>
<th>Caudal peduncle</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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</tbody>
</table>

**c. Presence of fishing nets on the animal**

<table>
<thead>
<tr>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tbody>
</table>

Fishing nets have been preserved?

<table>
<thead>
<tr>
<th>YES</th>
<th>NO</th>
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</thead>
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<tr>
<td></td>
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</table>

**d. Penetrating wounds**

<table>
<thead>
<tr>
<th>YES</th>
<th>NO</th>
<th>ND</th>
<th>NE</th>
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</thead>
<tbody>
<tr>
<td></td>
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</tr>
</tbody>
</table>

Describe gunshot wounds, puncture wounds, from harpoon, etc.

**e. Mutilations**

<table>
<thead>
<tr>
<th>YES</th>
<th>NO</th>
<th>ND</th>
<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
</tbody>
</table>

Describe ctus, tears, cracks in the body wall, missing appendages, etc.

**f. Hemorrhages and hematomas**

<table>
<thead>
<tr>
<th>YES</th>
<th>NO</th>
<th>ND</th>
<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Describe extension and area.

**h. Post-mortem damage from scavengers and opportunists**

<table>
<thead>
<tr>
<th>YES</th>
<th>NO</th>
<th>ND</th>
<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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</tbody>
</table>

Describe extension and area.
### 3. INTERNAL EXAM

**a. Sub-epidermal hemorrhages**

<table>
<thead>
<tr>
<th>YES</th>
<th>NO</th>
<th>ND</th>
<th>NE</th>
</tr>
</thead>
</table>

Describe extension and area.

**b. Fractures**

<table>
<thead>
<tr>
<th>YES</th>
<th>NO</th>
<th>ND</th>
<th>NE</th>
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</thead>
</table>

Describe.

**c. Content of airway and lung**

<table>
<thead>
<tr>
<th>AIR</th>
<th>FLUID</th>
<th>FOAM</th>
<th>ND</th>
<th>NE</th>
</tr>
</thead>
</table>

Describe lungs’ appearance (heavy, consolidated areas, color variations, etc.) and airway’s content.

**d. Stomach content**

Describe stomach content, amount, presence of parasites and foreign bodies.

<table>
<thead>
<tr>
<th>Stored in frozen</th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
</table>

**e. Histopathology**

<table>
<thead>
<tr>
<th>YES</th>
<th>NO</th>
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</table>
### 3. INTERNAL EXAM

<table>
<thead>
<tr>
<th>f. Presence of macroscopically visible lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>YES</td>
</tr>
<tr>
<td>Describe.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>g. DIAGNOSTIC HYPOTHESIS:</th>
</tr>
</thead>
</table>
8. GAS SAMPLING

<table>
<thead>
<tr>
<th>ID</th>
<th>SPECIES</th>
</tr>
</thead>
<tbody>
<tr>
<td>DATE OF DEATH</td>
<td>DATE OF NECROPSY</td>
</tr>
<tr>
<td>CONSERVATION CODE</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>FRESH OR FROZEN</td>
<td></td>
</tr>
<tr>
<td>STRANDED ALIVE</td>
<td></td>
</tr>
<tr>
<td>STRANDED DEAD</td>
<td></td>
</tr>
<tr>
<td>FOUND DEAD</td>
<td></td>
</tr>
</tbody>
</table>

**GAS SCORE**

| SUBCUTANEOUS VEINS<sup>1</sup> | |
| VENOUS PLEXUS LOIN-CAUDAL<sup>1</sup> | |
| MESENTERIC VEINS<sup>2</sup> | |
| CORONARY VEINS<sup>2</sup> | |
| SUBCAPSULAR EMPHYSEMA<sup>2</sup> | |
| TOTAL | |

1. **SCORE**

<table>
<thead>
<tr>
<th>SCORE</th>
<th>DEFINITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No gas bubbles / Presence of congested veins</td>
</tr>
<tr>
<td>1</td>
<td>Occasional presence of gas bubble</td>
</tr>
<tr>
<td>2</td>
<td>Some bubbles / Small discontinuities along the vessel’s course</td>
</tr>
<tr>
<td>3</td>
<td>Abundant and large discontinuities along the flow in the vessel, but not only filled with gas</td>
</tr>
<tr>
<td>4</td>
<td>Moderate presence of gas bubbles within a specific vein</td>
</tr>
<tr>
<td>5</td>
<td>Abundant presence of gas bubbles</td>
</tr>
<tr>
<td>6</td>
<td>Complete sections of the vessels are filled with gas</td>
</tr>
</tbody>
</table>

2. **PUNTEGGIO**

<table>
<thead>
<tr>
<th>PUNTEGGIO</th>
<th>DEFINIZIONE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No gas bubbles</td>
</tr>
<tr>
<td>1</td>
<td>Low presence of gas bubbles (UN only organ involved)</td>
</tr>
<tr>
<td>2</td>
<td>Moderate presence of gas bubbles (MORE THAN ONE organ involved)</td>
</tr>
<tr>
<td>3</td>
<td>Abundant presence of gas bubbles (MANY organs involved)</td>
</tr>
</tbody>
</table>

SUBCAPSULAR EMPHYSEMA LOCALIZATION: _____________________________
<table>
<thead>
<tr>
<th>N°</th>
<th>LOCALIZATION</th>
<th>METHOD</th>
<th>VOLUME (mL)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Subcutaneous veins</td>
<td>Insulin siringe</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Venous plexus loin-caudal</td>
<td>Insulin siringe</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Coronary veins</td>
<td>Aspirometer</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Heart – Right ventricle</td>
<td>Aspirometer</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Heart – Left ventricle</td>
<td>Aspirometer</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Aorta</td>
<td>Aspirometer</td>
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</tr>
<tr>
<td>7</td>
<td>Polmonary trunk</td>
<td>Aspirometer</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Mesenteric veins</td>
<td>Insulin siringe</td>
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<tr>
<td>9</td>
<td>Proximal intestine</td>
<td>Insulin siringe</td>
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</tr>
<tr>
<td>10</td>
<td>Middle intestine</td>
<td>Insulin siringe</td>
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</tr>
<tr>
<td>11</td>
<td>Distal intestine</td>
<td>Insulin siringe</td>
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<tr>
<td>12</td>
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<td>20</td>
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# 9. STANDARD SAMPLES

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Life History</th>
<th>Genetics</th>
<th>Contam.</th>
<th>Histo.</th>
<th>Morbilli</th>
<th>Brucella</th>
<th>Biotox</th>
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<tbody>
<tr>
<td>Adrenal (R)</td>
<td>(Frozen or fixed)</td>
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<tr>
<td>Aqueous humor</td>
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<td>Bladder</td>
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<tr>
<td>Blood/Serum</td>
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<td>Intestine</td>
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<td>Kidney (R)</td>
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<tr>
<td>Mesenteric Lymph.</td>
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<td>Milk/Mammary Discharge</td>
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<td>Oral Mucosa</td>
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<td>Pancreas</td>
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<td>Stomach Contents</td>
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<td>Tracheobronchial Lymph.</td>
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<td>Urine</td>
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<td>Uterus</td>
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</tr>
</tbody>
</table>
FIRST AID
AND
MANAGEMENT PROCEDURES
OF SEA TURTLES

Antonio Di Bello
DVM

Olimpia Lai
DVM, PhD
Sea turtles are exposed to many threats, from natural phenomena to anthropogenic factors. The diseases related to human activities are of many types: in the Mediterranean region the most frequent injuries result by accidental interaction with fishing activities. Very often, turtles are accidentally victims of long-lines, getting serious injuries in their digestive tract for hooks and lines ingestion, or damages in their fins for constriction by lines that can develop in necrosis and mutilation of limbs. Nets cause serious damages of limbs and external parts, and drowning syndrome caused by forced prolonged dives.

Other serious pathological events are related to accidental collisions with boats; contact with hydrocarbons and other pollutants discharged into the sea or by ingestion of floating waste (bags, bottles and other plastic items, fishing gear, etc..) cause poisoning and injuries.

Other accidents cause illness related to environmental, nutritional or metabolic factors (cold stunning, deficiency diseases, infectious and parasitic diseases, etc..).

In case of a stranded, drifting or accidentally caught sea turtle, the first aid provides a series of procedures really important to improve the recovery success.

First of all, it’s important to place the patient in a plastic container, dimensioned for the animal’s size and functional for the transfer, which allows the patient to maintain a ventral position avoiding compressing it or incorrect positions of limbs and head.

It is important to check the presence of foreign bodies that may block the upper respiratory tract of the animal, affecting breathing. In the tank the turtle lies on the plastron; the tank bottom should be covered by foam rubber or sponge or a towel.
folded in several layers.

Before moving a turtle in a rehabilitation center, it is important to record fresh identification data and information about finding. In particular, to note date and place of recovery, identification of the species and measurements of the curved carapace length (CCL n-t), general conditions of the animal and how it was found (accidentally caught by long-lines, nets or trawling, floating or washed up, etc.). During the transfer to the rescue center it’s important to keep the head, neck, carapace and flippers moist or wet. Covering the body with a cloth soaked in sea water may be the easiest way to do so (figure 1).

**Figure 1:** The turtle is placed in a plastic container on a towel folded in several layers. The body is covered with a wet cloth.

This method is easy and efficient if the ambient temperature is above 20°-22°C and if the trip is short; if the ambient temperature is low, a cold cloth might remove heat
from the body of the animal, which can be quite dangerous especially in hypothermic and debilitated animals. To avoid this fact, it is useful to wet constantly the animal with small quantities of warm water (28°-30°C). Of course, it is important to control the amount of water in the bottom of the tank, as if too much, it could compromise the breathing of debilitated animals.

During transport, it is appropriate to reduce stress for the animal: keep it in shadow, avoid noise, cover and protect its eyes, do not limit its ability to breathe properly, and avoid contact with other animals. In addition, it is requested to avoid animal movements into the tank, maintaining it in a normal ventral position without compressing it or assuming limbs and head abnormal positions (figure 2).

![Turtle](image)

**Figure 2:** The turtle has taken an abnormal and dangerous position of the head that makes breathing difficult.

Once at the rescue center, the animal receives the first clinical evaluation and the first aid. A reactive animal can be immediately placed in a tank with water, checking
if it breathes normally, otherwise in case of doubt, it is better to maintain the animal in a small amount of water, keeping the water level far below the nostrils. For turtle and people safety, it’s important to follow some correct procedures in order to raise, pull out of the tank and move a turtle. Depending the size, the animal is carried by one or two people. A single person can keep firmly the animal with one hand below the rear edge of the carapace, in correspondence of the caudal shield, and the other hand firmly to the cranial margin of the carapace, in correspondence with the nuchal shield (figure 3).

Another method for a single operator is to keep the animal with the plastron on his forearms, maintaining the opposite fin with his hand on the scapular-humeral region, tightening the animal to him, and with the other hand holding the turtle below the rear edge of the carapace (figure 4). This method is effective to reduce
stress to the animal but suitable only for individuals of less than 15 kilograms, otherwise it could be dangerous for the operator.

If the animal is large and heavy, two operators can move the animal from both sides, with their hands positioned cranially and caudally of the carapace (figure 5).

Figure 4: Another correct way to take a turtle.

Figure 5: The proper way to take the turtle in two persons.
Always we must absolutely avoid taking or lifting a turtle by its flippers. For a general evaluation, the animal is placed on a soft surface, keeping the body surface constantly wet.

The clinical observation assesses very carefully:

- nutritional conditions
- skeletal development
- muscle tropism and tone
- appearance of the skin and external surfaces of carapace, plastron, and head
- inspection of natural openings and external mucous
- respiratory capacity and mode
- temperature
- sensorium level and reflexes
- strange attitudes
- swimming and floating mode

The nutrition status can be easily evaluated by inspection and palpation of subcutaneous tissue of neck region and soft tissues that surround the proximal part of limbs. In subjects presenting good nutrition, soft tissue at the base of neck, axillary and inguinal regions are generally prominent to palpation, showing soft-elastic consistency due to the presence of adipose tissue (figure 6). In animals undernourished, axillary and inguinal regions appear sunken, the skin around the neck is not very elastic and the below muscles become evident (figure 7).

Tone and muscle tropism can be assessed through examination of front flippers: under normal conditions the proximal parts of limbs are surrounded by strong
muscle bellies; in debilitated animals, tone and tropism muscle may appear really reduced and the palpation of limbs allows to perceive easily the skeletal structures below the skin, sparsely covered with muscle structures.

Figure 6: Turtle in a good nutrition status: it is evident abundant fatty tissue under the skin of the neck and axillary region.

Figure 7: Turtle in a poor state of nutrition.
The inspection of skin and of external surfaces of the animal is fundamental and must be done very carefully. First of all, it is necessary to evaluate the presence of ectoparasites, their localization and extension, as well as the identification of types (barnacles, limpets, leeches, algae, etc..) (figure 8). If present in excessive amounts, they may be a first sign of the turtle’s discomfort because they tell us that the animal has recently been moving slowly. Because ectoparasites are very tenaciously attached to the surface of the animal, their removal should not be done mechanically using knives, chisels or other tools that could be traumatizing, as they risk to severely damage skin and shield. For a removal without trauma, it is enough to maintain the patient for two or three days in fresh water, resulting in death and detachment of all ectoparasites. Of course this is possible after to complete clinical evaluations and determine that the turtle can swim in a tank.

Figure 8: Turtle with many barnacles and limpets attached to the plastron.
The analysis of external wounds helps to understand cause and time of them. Injuries located on skull and carapace are more frequently due to accidental trauma with boats or rocks during storms, if the animal was weakened (figure 9).

![Image of turtle with injuries](attachment:image.jpg)

**Figure 9:** Wide and deep injury of the carapace caused by the impact with a boat.

Very serious neck and limbs injuries can be caused by lines, ropes, plastic bags or other foreign waste floating in the sea (figure 10). Skin lesions, localized or diffuse, can be caused by primary and secondary bacteria and fungi growth, in this case it is important to carry pads and skin scrapings for identification of causative agent. External inspection of the mouth allows to evaluate the presence of lines or other foreign bodies, wounds and sores in the mouth corners, as the poor status of nutrition, generally connected with the presence of a hook or a line ingested for a while (figure 11).
Figure 10: Tissue necrosis of the filipper caused by fishing lines constriction.

Figure 11: Long line coming out of mouth and has caused serious injury and necrosis by compressing the lower jaw.
The deep inspection of the oral cavity helps to detect hooks anchored in the proximal part of the esophagus, inflammations and ulcerations of the mucosa or other injuries; their nature should always be determined through the execution of sterile swabs and laboratory tests (figure 12).

![Image](image.jpg)

**Figure 12**: The deep inspection of the oral cavity reveals a bleeding in the esophagus.

The external inspection of cloacal opening evidence if there is a line or a prolapse or edema of the surrounding soft tissues (figure 13).

When out of the water or in a stressful situation, turtles may alter the frequency and manner of their breathing, for this it is important to observe if inhalation and exhalation phases look limited, or they produce breath sounds or biological material or foreign objects from the mouth or nostrils (figure 14).
Figure 13: Slight prolapse of the cloaca and edema of the surrounding soft tissues caused by a longline crossing the intestine.

Figure 14: Foam spilling from the nostrils due to a pulmonary edema, caused by a prolonged apnea in the trawler net.
The measurement of the internal temperature can be recorded by infrared thermometers or an endocloacal probe; for an efficient metabolism, the body temperature should not be lower than 18 °C. During winter, it is very frequent to recover specimens in hypothermia, or cold-stunning, this often affects small turtles as a result of sudden reduction of temperature, for sudden weather changes. In these circumstances animals are slaughtered, lethargic, bradycardic and bradipnoic. As hypothermia causes lowering of metabolism, reptiles are not able to respond to any drug treatment, so it is necessary to maintain their body temperature at an optimum level to ensure a good metabolism, between 25 ° and 30 °C.

The assessment of the sensorium status is carried out through reflex reactions and physiological reactions. Reflection tests are retraction of neck and fins, opening of the mouth, blink reflex, as cloacal and tail ones. Normally after a slight traction of the head or of the end of a fin, the animal instinctively retracts the part. Another reaction is stimulated by lifting with caution the top of ranfotec: the animal often reacts instinctively arching high the neck and opening the mouth, this reaction is also very useful to inspect the oral cavity. Blink reflex is stimulated by tapping lightly with a soft object (eg. a wet cotton swab) the lateral or medial margin of the eyelids: to this stimulus the animal normally reacts with the quick closure of its eyelids. In normal conditions, a gentle introduction of the tip of a finger or a wet cotton swab into the cloaca or moving laterally its tail, the animal reacts by contracting the cloacal sphincter and ventral bending forward the tail (figure 15). When one or more of these reflexes are delayed, not evident or even absent, this is a sign that the animal is in severe illness and prostration.
These important assessments are followed by morphometric and weight measurements.

If no clinical signs are evident, the patient is checked in its tank, in order to assess its breathing, movements in water, attitude and ability to dive and to emerge without difficulty.

The next very important step is the sampling and analysis of biological samples to complete the study of the state of health of the animal.

To take samples of biological material but also in many phases of clinical examination, it is important to restrain the turtle so that it is not damaged, that the animal does not cause operator injury and even that it does not receive too much stress. To that end, first of all it is appropriate to cover the eyes of the animal so that

Figure 15: Evaluation of cloacal reflex with a wet cotton swab.
it is quieter. To do this you can use a cotton sock, preferably dark colored, which is threaded on the head, or you can wrap the head with a self-adhesive elastic bandage, taking care not to cover the nostrils. To restrain properly the turtle on the examination table, the operator must stand behind the animal and take the front flippers firmly at the humerus-radio-ulnar joint. Resting his elbows on the carapace, the operator press down slightly and at the same time with hands keep the front flippers pulled back and close to the carapace (figure 16-17). In larger animals is appropriate that the operator lean his bust on the dorsal part of the carapace, and with his weight keep firm the animal.

Figure 16: Positioning of the operator to properly restrain the turtle (frontal view).
Biological samples and analysis include blood samples for biochemical assessment and emocromocitometry by cervical venous sinus or jugular veins. These procedures require a minimum of experience and manual skills. The use of the cervical venous sinus is realized containing the animal with the neck extended and slightly down. The needle connected to the syringe is introduced into the dorsal surface of the neck, in the paramedian position between cervical spine and cervical biventer muscle. The needle is introduced with an angle of about 45-60 degrees in the skin, either on one of the two sides, in an area between the neck and cranial edge of the carapace (figure 18).

Figure 17: Positioning of the operator to properly restrain the turtle (lateral view).
The jugular vein sample is obtained containing the animal with the head in extension and inserting the needle of one of the two sides of the neck, in a cranio-caudal direction and with an angle of approximately 30 degrees to the skin. The needle is inserted in the depression of the neck just below the transverse cervical muscle (figure 19).

By the same way, when needed, fluids and drugs can be administered in bolus or with continuous controlled infusion.

When intravenous route is difficult or impossible to reach, fluids can be dispensed by via intracoelomic, subcutaneous or intraosseous bridge between carapace and plastron.

Figure 18: Puncture of cervical venous sinus.
Fecal samples can be obtained from the bottom of the tank, immediately after defecation, with a highly textured screen; an amount of feces can be taken directly from the rectum with a digital exploration of the cloaca.

Oral, conjunctival and skin samples are taken for virological, bacteriological and mycological analysis.

Actually sea turtle sedation and anesthesia are obtained using various protocols. During sedation and anesthesia even if for short time, it is important to provide for the intubation until complete awakening. During the first steps of anesthesia and awakening, it is important to check the breathing mechanically or manually (2-4 breaths per minute) until complete spontaneous use of the function. Before recovering the turtle in its tank, the animal has to confirm the ability of swimming.
and breathing.

Except particular cases, it is appropriate to feed the patient daily, still immediately after surgery. In case of lack of appetite or prolonged fasting, it’s better to force feed the patient, until the spontaneous feeding. This procedure is realized by introducing an oral gastric probe to administrate homogenized food. The forced tube feeding is a very stressful procedure for turtles and operators, and risks of damages to oesophagus (figure 20). To assure a less traumatic feeding procedure, it’s recommended to use an oesophagogastric probe inserted through a temporary stoma, realized in the lateral surface of neck (figure 21).

Figure 20: Execution of forced tube feeding in a small turtle.
Figure 21: Daily force feeding through an oesophagostomy tube.
Bibliography


Sea turtles handling and first aid

STRANDED TURTLES:

- Put the turtle in a plastic container, with a soft surface under the plastron (mattress, life buoy, folded towel..);
- During summer use a wet towel to cover the carapace and keep head and eyes wet; keep it in shadow to avoid overheating;
- During winter, use a dry towel to cover it and keep it in a warm place.

- Hold the turtle by its carapace as in the images.

- If the turtle has bloody wounds, use a bandage or a clean towel to cover them and minimize handling.

DO NOT:
- Hold the turtle from FLIPPERS.
- Put the turtle on the CARAPACE.
- Get too close to the MOUTH: pay attention to bites!
- Remove EPIBONTS by hand.

Minimize stress!
- put a wet towel on the head in order to cover the eyes; leave nares free.
- keep people away from the animal, avoid excessive noise.
SEA TURTLES ADRIFT:

- Use a dip-net to board the turtle.

**DO NOT:**
- Bring the turtle in by pulling the line

- If the turtle has been hooked:
  - cut the line as close as possible to the mouth;
  - take it to the Rescue Center.

**DO NOT:**
- Try to pull out the hook
- Pull the line from the mouth or from the cloaca

- If the turtle has been caught during fishing operations and it looks comatose or dead:
  - put it in a slope position (lift hindquarters of about 20-30 cm) until it get active again (at least 4 hours);
  - take it to the Rescue Center.

**DO NOT:**
- Put the turtle in the water if it still looks comatose
Stranded cetaceans handling and first aid

AIM: reduce stress, give relief from hot, sun and wind until the arrival of first aid group

Approaching to the animal

✓ Approach the animal quietly and slowly from one side or in front. Avoid noise, lights and movements.
✓ Be careful to mouth and tail. Mouth, body and tail should be blocked simultaneously by personnel.
✓ Social and pelagic species are subject to stress.
✓ Assess the presence of skin wounds and look for fishing nets around the animal or in the mouth.
✓ Monitor: Respiratory rate: 4-8 breaths/minute
  Interaction with the external environment
  Abnormal behavior: ➔ In the water: the animal is swimming rotated on circle, shows abnormal diving, etc.
  ➔ On the shore: tremors, convulsions, unusual sounds, etc.

Alive cetaceans close to the shore

With no evident injuries or physical and behavioural changes:
  - Constant monitoring with no intervention
  - Try to approach the animal in order to assess if it runs away

✓ If environmental condition could be dangerous ➔ Try to push the animal toward the high sea
  - If is possible, use inflatable rafts
  - If using motorboats take care of the propeller
  - Approach the animal slowly from one side
  - Maintain safe distances
  - Try to make noise between the animal and coastline (for example: create a human chain making noise with metal objects). If unsuccessful after short time, stop it and try after some time
  - Note any altered behavior

✓ If the animal is injured/sick ➔ any refloating effort could delay stranding:
  - Evaluate the option to take the animal close to the shore for medical evaluation
  - Contact an expert veterinarian or a first aid team
Stranded cetaceans

✓ Reduce manipulation and stressful condition, in particular in pelagic species.
✓ Maintain people at safe distance.
✓ Approach the animal from one side or in front.
✓ Do not touch eyes, blowhole and genital area (protect blowhole and eyes with saline solution or lubrificant).
✓ Do not use fins to move the animal.
✓ Dig holes under pectoral fins.
✓ If is possible use inflatable of foamy mattress on rocky beaches.
✓ The animal must be positioned on its belly.
✓ Avoid overheating (also in cloudy days) by:
  - Wet towels
  - Shadowing the animal
  - Suntan or hydratant cream (zinc dioxide)

✓ Avoid cooling (especially during winter):
  - Use mineral oils or cream on fins in small and emaciated animals to avoid thermic dispersion

Moving the animal

✓ Do not use ropes.
✓ Do not pull an animal without towels or in presence of rocks
✓ Do not pull from fins and/or tail.