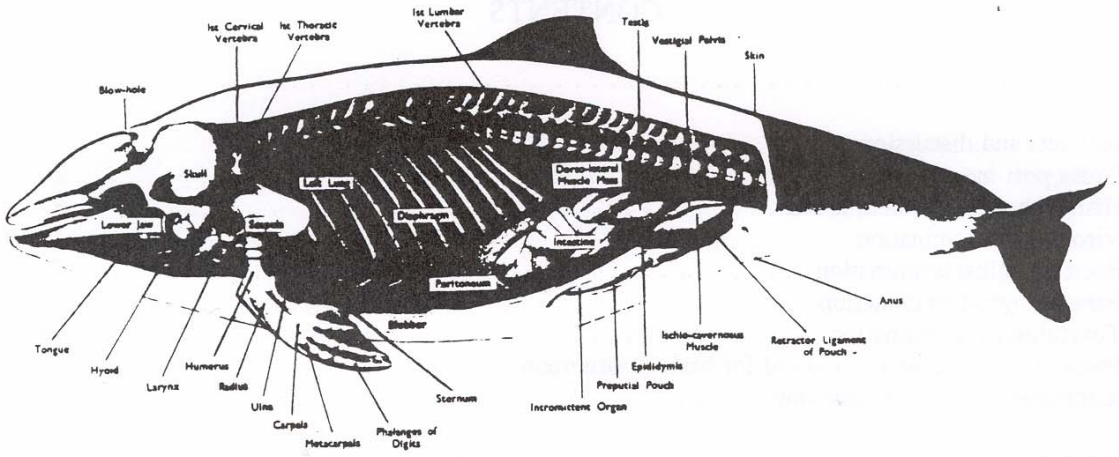
Proceedings of the first ECS workshop on

CETACEAN PATHOLOGY:

DISSECTION TECHNIQUES

AND TISSUE SAMPLING



Leiden, The Netherlands, 13-14 September 1991

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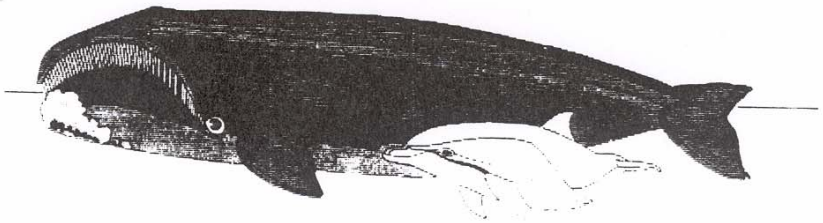

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PROCEEDINGS OF THE FIRST EUROPEAN CETACEAN SOCIETY WORKSHOP ON CETACEAN PATHOLOGY: DISSECTION TECHNIQUES AND TISSUE SAMPLING, LEIDEN, THE NETHERLANDS, 13-14 SEPTEMBER 1991

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Cover figure from *Handbook of R.H. Burne's Cetacean Dissections*, British Museum (Natural History), London, 1952.

PREFACE

Intrigued by the question why cetaceans strand, and realizing that studying carcasses of stranded animals was one of the only ways to learn more about their biology, scientists started recording information about strandings many years ago. At first they recorded information such as species, sex, length, location and date of stranding. This increased our knowledge greatly about the distribution and population biology of cetaceans.

As more countries started to record similar information, it became important to standardize the methods of taking measurements and collecting samples from cetaceans. Only by standardizing methods is it possible to compare results of studies of strandings from different geographical areas. Norris (1961) edited an article on carrying out body measurements on small cetaceans which is often still used as a standard for this. With advances in scientific techniques and shifting priorities of research, changes have taken place in the required methods of examination. This led to a workshop, held by the ECS in Cambridge in 1988, to standardize the body measurements and life history samples taken of stranded harbour porpoises, which are widely distributed in European waters.

In contrast to examination related to the life history of a stranded animal, the pathological examination, that is the examination to detect disease, both infectious (e.g. caused by viruses or bacteria), parasitic, and non-infectious (e.g. caused by trauma, starvation, or biotoxins) has been neglected in the past. Although there are historical examples of mass mortalities in marine mammals (Laws and Taylor, 1957; Gallacher and Waters, 1964), and more recent ones in North American waters (Anon. 1991), it took until the late eighties, with an epizootic in common seals in north European waters (Osterhaus *et al.*, 1988) and in striped dolphins in the Mediterranean (Domingo *et al.*, 1990), to make the scientific community and the funding bodies realize that disease has a part to play in the population dynamics, and therefore in the population management, of free-living cetaceans.

Secondly, there is also a strong concern, albeit as yet not based on hard scientific facts, that the pollution of their environment is causing cetaceans to be more susceptible to disease. In conjunction with taking samples of tissues for toxicological analysis, pathological examination may help to establish the effect of environmental contaminants on the immune system.

Thirdly, pathological examination of stranded cetaceans may help to establish if they died after becoming entangled in a fishing net, and so help to determine the effect of interactions with fisheries on the population dynamics of cetaceans.

As pathological examination is being incorporated in the protocols of a number of groups working on cetacean strandings throughout Europe, more attention is being given to cetacean pathology. Therefore, it was decided to organize a workshop on cetacean pathology. The goals of this workshop were:

1) to bring together the people carrying out examinations of stranded cetaceans in Europe;
2) to standardize the basic pathological examination of small cetaceans, integrated with measuring and taking tissue samples for life history and toxicology studies;
3) to demonstrate in practice the examination of a harbour porpoise carcass.

The workshop was held in Leiden on 13-14 September 1991, hosted by the Leiden National Museum of Natural History. More than 50 people from 10 countries in Europe and from the USA took part (Appendix 1). Two harbour porpoise carcasses were kindly made available by the museum for the demonstration of postmortem techniques.

Presentations were given on various aspects of postmortem examination. The emphasis was on the pathological examination, as it was thought that the standardisation of basic techniques for life history and toxicology studies was already more established. Next, a standard protocol for the basic examination of small cetacean carcasses was agreed upon. Based on this, a protocol (Appendix 3) and recording form (Appendix 4) were written.

We are grateful to the Leiden National Museum of Natural History for providing the venue of this workshop, and especially to Marjan Addink for helping to organize it; to Genevieve Desportes, Nuria Calzada, Peter Evans, Torsten and Anne Mörner for their comments on the manuscript; and to Terry Dennett and Mandy Walton for their help with the lay-out.

We hope that these proceedings will be of use to those people carrying out post mortem investigations on cetaceans, and that the basic protocol will be widely adopted. If that happens, it will help to provide a view of the population biology of cetaceans that is not limited by the borders of countries.

Thijs Kuiken
Manuel García Hartmann

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GROSS POSTMORTEM EXAMINATION

John Baker

The gross postmortem examination of small cetaceans was described, with particular reference to lesions commonly found and anatomical features specific for cetaceans. Baker stated that there is no right or wrong way of carrying out a postmortem, so long as the complete body is examined. In his opinion it is of no use whatsoever to do a postmortem unless a detailed record is kept of what one has found. The method used at the University of Liverpool generally follows the "UK standard guidelines for postmortem examination and tissue sampling of cetaceans", and information is recorded on the "UK standard cetacean postmortem report form". Copies of these are available from the ECS pathology working group.

Discussion

Although organs are routinely weighed in some places in Europe, the value of weighing organs is limited from a pathological point of view, mainly due to lack of reference values and variation in organ weight caused by variable degrees of congestion. It was generally considered that the information gained from weighing organs was not sufficient to justify including it in the minimum protocol, with the exception of weighing the testes.

With regards to the measurement of the blubber thickness, it was agreed to measure this just behind the dorsal fin, as agreed at the ECS harbour porpoise workshop held at Cambridge in 1988. However, it was generally considered that the reliability of the blubber thickness to evaluate the nutritive state was poor. The percentage of extractable lipid in the blubber seemed to be more suitable to quantify the nutritive state. Other parameters mentioned to evaluate the nutritive state were the presence of subcutaneous fat, the total blubber weight and the fibrous quality of the blubber.

In Baker's opinion one should open the intestine, wash the contents in a bucket, and sieve the washings through a mesh of not more than 1 mm diameter. By doing this the number of otoliths, bones, and parasites is markedly increased in comparison with just examining the opened intestine.

In the experience of several participants, one should beware of taking too many samples, as storage space is often limited.

HISTOPATHOLOGICAL EXAMINATION

John Baker

Methods for collecting and fixing samples for histopathological examination were described. The samples for histopathological examination taken at the postmortem examination of cetacean carcasses at the University of Liverpool were listed. These are generally the same as described in the "UK standard guidelines for postmortem examination and tissue sampling of cetaceans", and the "UK standard cetacean postmortem report form". Copies of these are available from the ECS pathology working group.

From Baker's experience it was clear that the more tissues were examined microscopically, the more pathological lesions would be found. In those cases where lesions were visible with the naked eye, histological examination helped to differentiate different types of lesions. In other cases, grossly normal organs were found to have pathological lesions when examined microscopically. In his opinion, it is also useful to record histopathological findings of lesions of which the pathogenesis is poorly known, to help to understand more about diseases in cetaceans.

Discussion

Besides detecting pathological lesions invisible to the naked eye, the histological examination of apparently normal tissues helps to build up knowledge on the physiological microscopic appearance of cetacean tissues for comparative purposes.

Histological examination of tissues involved in the body defence appears to be meaningful in the context of postulated pollutant- induced immune suppression. Lymph nodes were found to be difficult to evaluate, as their microscopic appearance varies with functional state and location. Apparently, the drainage areas of some lymph nodes do not always correspond with those of land mammals. The thymus was found not to be suitable for an easy evaluation of immune function and therefore was not included in the minimum protocol.

The histological examination of the lungs, even if grossly normal, often reveals infiltration with polymorphonuclear leucocytes.

VIROLOGICAL EXAMINATION

Albert Osterhaus

Before samples for virological examination are taken from aquatic mammals that have stranded or have been admitted for autopsy, it is important to define why virological investigations are carried out in the first place. We may be interested to relate particular symptoms or lesions observed in one or more animals to a virus infection. In this case the sampling will focus largely on the affected organs or organ systems involved and techniques will be applied that may favour the detection of the virus expected to be present. The sampling procedures will then be similar to those used for virological procedures used for humans and other animals.

As an example, in the case of acute respiratory infections, samples from the affected upper respiratory tract and lungs and the corresponding lymph nodes will be collected. In the case of focal lesions like pox, warts, vesicles or ulcers, material from these specific areas should be taken. When viraemia is suspected to have occurred, preferably non-clotted blood should be collected in heparin from the animal just before or just after it has died.
The chances of identifying a virus infection in such clinical specimens depend directly on the quality and the timing of the sampling procedure and level of specialisation of the virological laboratory involved. Often the success of this approach, focusing on affected organs or lesions in an early phase of the investigation, is also related to the availability of anamnestic and clinical data. Whenever possible, these should include epizootiology and also knowledge about the viruses that have already been identified and may be expected to occur in the species involved.

An overview of the present knowledge on virus infections of pinnipeds and cetaceans is given in Table 1. If no clinical or anamnestic data are available and the histopathology does not offer any indications, or if a more systematic and thorough inventory is required, a systematic sampling of all organ systems or a selection thereof may be required. No general recommendation about such a selection can be given since they depend entirely on the type of virus infection and organ systems one is most interested in.

The techniques for virus identification can roughly be divided into those that demonstrate the presence of the whole replication-competent virus, physical virus particles or their components (such as specific proteins or nucleic acids), and those that identify specific antibodies. The major advantages and disadvantages of the respective techniques used are given in Table 2.

Samples for virus isolation procedures, which are often required when little information is available about the viruses that may occur in a particular species, should always be collected as soon as possible after death of the animal, or if possible even before the animal has died. In the case of a stranded animal this is usually a major problem.

Table 1: Virus infections of marine mammals (partly from Visser *et al.*, 1991)

Virus family	Virus	Aquatic mammal species	Demonstration (year)	Number infected animals	Related deaths	Clinical symptoms
Adenoviridae	Sea Lion Hepatitis Virus	California sea lion	1978 1980	5 1	5 1	diarrhea/anorexia/abdominal pain/posterior paresis/ polydipsia/photophobia
		Sei whale	1977	1	1	unknown
Herpesviridae	Alphaherpesvirinae Phocid Herpesvirus-1	Harbour seal	1984 1988	23 several	11 unknown	nasal discharge/inflammation of oral mucosa/vomiting/
		California sea lion	1986	1	unknown	diarrhea/fever/coughing/ anorexia/lethargy
	Uncharact. Herpesvirus	Beluga whale	1989	1	unknown	focal dermatitis
Poxviridae	Seal poxvirus Parapoxvirus	Harbour seal California sea lion Grey seal Northern fur seal South American sea lion	since 1969	several " " " "	unknown " " " "	pustular proliferative skin lesions
		Bottlenose dolphin White sided dolphin Harbour porpoise		several " "	several " "	tattoo-like hyperpigmentated skin lesions/generalized disease
	Orthopoxvirus	Grey seal		1	unknown	unknown
Picornaviridae	Picornavirus	Harbour seal	1988	several	unknown	unknown
		California grey whale	1968	≥ 1	unknown	unknown
Caliciviridae	San Miguel Sea Lion Virus	California sea lion Northern fur seal Northern elephant seal Pacific walrus Steller sea lion	since 1969	several " " " "	suspected " " " "	abortion/ ulcerative skin disease
		Atlantic bottlenose dolphin California grey whale Bowhead whale Sperm whale		several " " "	suspected " " "	
Orthomyxoviridae	Influenzavirinae H7N7 Influenza A virus H4N5	Harbour seal	1979 1982	> 600 many	> 400 > 60	hemorrhagic pneumonia
	H13N9/H13N2	Pilot whale	1984	> 1	suspected	sloughing skin/emaciation
		Striped whale	1984	1	"	respiratory infection
Paramyxoviridae	Canine Distemper-like Virus	Baikal seal	1987	≥ 8,000	≥ 6,000	fever/serous nasal discharge/ conjunctivitis/anorexia/ central nervous symptoms/ breathing problems/gastro-intestinal disturbances/ cutaneous lesions/secondary infections
	Phocid Distemper Virus	Harbour seal Grey seal Ringed seal Harp seal	1988 " " "	~ 20,000 several sporadic "	~ 17,000 unknown " "	
	Porpoise Morbillivirus Dolphin Morbillivirus	Harbour porpoise Mediterranean striped dolphin	since 1988 1990	several ≥ 600	unknown ~ 600	unknown respiratory disease/ secondary infections
Coronaviridae	Coronavirus	Harbour seal	1987	3	3	unknown
Rhabdoviridae	Rabies virus	Ringed seal	1980	1	1	pneumonia/enteritis/paralysis/ aggressive behavior/hydrophobia/extensive salivation
Retroviridae	Spumavirus	California sea lion	1986	1	unknown	probably: skin lesions

Table 2: Identification of virus infections

	time	labour	sensi-tivity	speci-ficity
1. "Typical" clinical signs				
- thorough clinical examination	▌	▌	▌	▌
- thorough anamnestic evaluation	▌	▌	▌	▌
2. "Pathognomonic" changes in tissues or cells				
- macroscopical evaluation	▙	▌	▌	▌
- histology	▬	▌	▌	▙
- EM	▚	▌	▌	▬
3. Direct detection of viral presence				
- EM or immune EM (IEM)	▙	▙	▙	▬
- virus isolation (+ characterization)	▬	▬	▬	▬
- antigen detection (IFA, IPA, ELISA....)	▌	▌	▬	▬
- nucleic acid detection (*in situ* hybridiz., PCR)	▚	▌	▬	▬
4. Detection of specific antibodies				
- paired serum samples (serology....)	▛	▌	▛	▬
- IgM (IFA, ELISA....)	▌	▌	▚	▬

General principles of aseptic sample collection with proper caution for zoonotic infections should always be followed. For most sampling procedures, shipment in special containers in the correct transport medium, lasting less than 24 hours under permanent cooling (0-4°C) is necessary. However, for certain viruses other conditions are more favourable. Again, no general guidelines for all cases can be given. If no immediate shipment can be arranged, it may be necessary to preserve the samples by freezing at -70°C. Higher temperatures may cause rapid loss of infectivity of certain viruses.

Samples for virus isolation should never be fixed in formaldehyde solution or any other fixative. Although certain procedures for specific antigen detection (ELISA, IFA) or detection of specific nucleic acid sequences (polymerase chain reaction, nucleic acid hybridisation) may still work on fixed tissues, fresh samples are always preferable.

Serological techniques are carried out with properly collected, non-haemolytic serum, blood or plasma samples, but under certain conditions these techniques can also be performed with haemolytic sera, body fluids or tissue extracts. If no IgM specific reagents are available, as currently is the case for most aquatic mammal species, only the presence of antibodies produced long before the moment of sampling can be demonstrated.

The most important advice to those who wish to identify a virus infection in an aquatic mammal, is probably to first contact the virologist, who will be responsible for the virological studies. A discussion at an early stage between the pathologist and the virologist about the sampling strategy to be followed and the conditions of the shipment, has usually proven to be crucial for the successful outcome of the virological investigations.

Finally it should be realized that the outcome will not only be determined by the skills of the clinician and the pathologist involved, but that also the expertise of the virological laboratory in the field of aquatic mammals may be crucial. The level of expertise and specialisation is determined in part by the availability of cell cultures of the relevant species, specific antibody preparations, probes and primers. The establishment of collaborative networks between pathologists and virologists should therefore be considered essential.

Reference

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Discussion

It was discussed how long after death a virus could still be detected in the carcass. If the carcass is maintained at about 4°C, virus isolation might be possible up to four days post mortem. Some methods of keeping the animal cool under field conditions were mentioned, of which the best seemed to be keeping the animal in cold water with ice. Antigen and nucleic acid detection are possible for longer than 4 days post mortem if the tissues are not too autolysed. If the carcass has to be stored frozen prior to postmortem examination, serum for antibody detection should be taken first.

Another point mentioned was that proving the presence of a virus (including virus isolation) does not necessarily mean that this virus is the cause of death.

Much discussion concerned the number of samples to be taken for virus isolation. Osterhaus argued that taking a long list of samples routinely only involves a small amount of work when compared to the rest of the autopsy, and is therefore worthwhile.

Morbillivirus was considered to be an important recent pathogen and there was a general feeling that all cetaceans in European waters should be screened for morbillivirus routinely, at least during the next few years. For this purpose, the minimum samples that should be taken are lung, brain, kidney, and serum. It should be realized that morbillivirus infection may cause immunosuppression, so that secondary infections with other pathogens may also be present in the carcass.

An important point raised in the discussion was that haemolytic blood - which cannot be separated into serum and cells - still may be useful for certain serological tests after ammonium sulphate precipitation. However, the antibody concentration in the blood decreases significantly if the animal has been dead for more than 5-6 days.

BACTERIOLOGICAL EXAMINATION

Stephen Macgregor and Thijs Kuiken

Introduction

Bacteria may cause disease in cetaceans, either independently or in combination with other factors such as parasitic infestations or malnutrition. There are a number of difficulties involved in interpreting the results of bacteriological examination of stranded cetaceans. Firstly, there is little

information on which bacteria occur in cetaceans, either as pathogenic or commensal micro-organisms. There are reviews on the bacteria isolated from cetaceans (Dailey, 1985; Howard *et al.*, 1983), both in captivity and in the wild. These reviews show the gaps in our knowledge of this subject. Secondly, the animals submitted have often been dead for some time, leading to postmortem changes in the tissues that may complicate bacteriological examination. Finally, it is difficult to compare studies carried out on cetacean strandings in the past because of the differences in tissues that were sampled and the techniques that were used.

Below, methods to carry out bacteriological examination on cetacean carcasses will be shown. Attention will be given to the problems of postmortem changes, and a standard protocol for both the basic and optimal bacteriological examination will be proposed.

Carcass storage prior to postmortem examination

After an animal dies, changes occur in the bacterial populations present. The number of a bacterial species may increase or decrease, one species may overgrow another species occurring in the same tissue, and tissues may be invaded by bacteria, either from outside the body, or from another site in the body. Therefore, a carcass should be examined immediately after it has died. Unfortunately, in the case of stranded cetaceans, the carcass is often only found some time after death, and there is often a lapse of time between when it is found and when bacteriological examination takes place. To minimise this lapse of time, it is important to have an extensive and efficient network for reporting cetacean strandings. If the ambient temperature is high, the carcass should be stored in a cool place out of the sun - preferably in a large refrigerator at 0-4°C - until postmortem examination can take place. This will slow down changes in bacterial populations. If possible, the abdominal cavity of the carcass should be slit open to allow more rapid cooling of the abdominal organs. Freezing has negative effects on the results of histological, bacteriological and viral examination, and only if it is known that the carcass has to be stored for more than a week before postmortem examination, is it preferable to freeze the carcass at -20°C.

Tissue sampling

To take samples for bacteriological examination, one can either take swabs or tissue blocks. Each of these methods has its own advantages and disadvantages, and the choice depends on the preference of the person carrying out the examination, and of the relevant laboratory.

To take a block of tissue, one should cut out a block approximately 4x4x4 cm using a sterile scalpel and forceps, and place this in a sterile container. Samples should be kept at 0-4°C until processing, which should be carried out as soon as possible.

For samples of intestine, one should tie off a loop of intestine approximately 10 cm long, cut it off using sterile instruments and place it in a sterile container. This should be kept at 0-4°C until processing.

To take a heart blood sample, one should disinfect the surface of the right ventricle, for example with 70% alcohol, and take a blood sample from the lumen using a sterile syringe and needle. One can also use a sterile pipette. In both cases, the blood sample should be stored in a sterile container at 0-4°C until further processing.

To take a swab, one should disinfect the surface of the organ, for example with 70% alcohol, incise it with a sterile scalpel, and place a sterile swab in the incision. The swab then can be inserted in a sterile tube with transport medium. Sterile swabs and containers with transport medium are available

commercially as packages. A suitable choice is a package for aerobes and anaerobes, containing for example charcoal transport medium. The swab should be kept at 0-4°C until processing.

Tissue smears

It is useful to make smears of tissue samples and to stain these for microscopical examination. In this way it is possible to quickly detect and to roughly identify micro-organisms in a sample. Smears are also important as a screening method for those micro-organisms, e.g. Chlamydiae, Rickettsiae, Mycoplasmata, and fungi, which may not grow on the standard media.

Useful stains are:
-Gram's method to differentiate between Gram-positive and Gram-negative organisms (Cowan, 1975).
-Ziehl-Neelsen's method (Acid-fast stain) to detect mycobacteria (Cowan, 1975).

Isolation media and atmospheric conditions

The following media are suggested for standard bacteriological examination:

Columbia Blood Agar
Columbia agar (Ellner *et al.*, 1966) plus 5% blood can be used for the isolation of bacteria at 37°C under both aerobic and anaerobic conditions.

Wilkins-Chalgren Agar
This medium (Wilkins and Chalgren, 1976) can be used for the isolation of obligate and facultative anaerobic or micro-aerophilic organisms. It is incubated at 37°C under anaerobic conditions.

Identification of bacterial isolates

The API system, by Biomérieux® (La Balme les Grottes, 38390 Montalieu Vercieu, France) can be used to identify Gram-negative rods. Two types of strips are used, 20E (for Enterobacteriaceae and other Gram-negative rods) and 20NE (for Gram-negative rods not belonging to the Enterobacteriaceae family). The ID232A strips can be used for the identification of anaerobic bacteria.

Gram-positive bacteria can be identified along traditional methods (Cowan, 1975; Buchanan *et al.*, 1975).

Standardised protocol: optimal procedures for bacteriological examination

In all circumstances one should attempt to examine a cetacean as soon as possible after death, and take measures to prevent postmortem changes along the lines discussed above. With regard to the samples to take and the methods to use for bacteriological examination, the protocol summarised in table 1 can be used.

Samples from heart blood, liver, kidney, jejunum, internal and external lesions of suspected bacterial origin should be inoculated on Columbia blood agar. Anaerobic incubation on Wilkins-Chalgren agar should be included as these sites offer the conditions for anaerobes to grow. Heart blood is included specifically to identify septicaemia; it appears to be a good sample site for bacteriological examination from a dead animal as it offers good protection from post-mortem invasion of bacteria.

Table 1: Standard protocol for optimal bacteriological examination

SITE	MEDIUM	TEMPERATURE	ATMOSPHERE
-internal lesions -external lesions -liver	Columbia blood agar	37°C	aerobic
-kidney -heart blood -jejunum	Wilkins-Chalgren agar	37°C	anaerobic
-lung	Columbia blood agar	37°C	aerobic

A lung sample, taken from the cranio-ventral part of the left lung - as this part of the lung is often the first to be infected - should be inoculated on Columbia blood agar and incubated aerobically. Anaerobic incubation is not necessary as one would not expect to find an anaerobic organism growing in the well-aerated lung tissue.

Smears from these tissue samples should be air-dried and stained with Gram's method. Lung samples and samples from tuberculous lesions should also be stained using Ziehl-Neelsen's method to detect any mycobacteria.

In addition to this protocol, one should use specific media if one suspects a bacterial species, on the basis of the gross postmortem examination or the tissue smears. For example, in case of an atypical pneumonia, samples can be taken for isolation of *Mycoplasma* species (Marmion, 1989).

Standardised protocol: minimal procedures for bacteriological examination

As a minimum, one should still attempt to take the following samples for bacteriological examination:
-any lesions with a suspected bacterial etiology should be sampled
-a sample of heart blood should be taken to detect a possible septicaemia
-as bacterial infection of the lungs is so common, one should also at least take a sample of lung tissue from the cranio-ventral part of the left lung.

Samples should be inoculated onto Columbia blood agar and incubated for 5 days at 37°C aerobically and anaerobically, except for the lung sample, which need only be incubated aerobically.

Smears from these tissue samples should be air-dried and stained with Gram's method. Lung samples and samples from lesions of suspected mycobacterial etiology should also be stained using Ziehl-Neelsen's method.

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Discussion

Because all internal organs are rapidly invaded by enteric bacteria, it is difficult to differentiate this postmortem invasion from sepsis. Spleen and heart blood may be the most suitable tissues to detect any bacteria causing sepsis.

It was mentioned that the bacterial flora of cetaceans may vary considerably according to geographic location and over time, in the same way as is known to be the case in seals.

For many bacteria isolated from cetaceans, it is not clear if they are pathogenic or not. It is usually necessary to correlate bacteriological findings with the results of histopathological examination to answer this question.

It was thought that Vibrio species and anaerobic bacteria may play an important role as pathogens.

Although sometimes difficult to determine by gross pathology, bacterial pneumonia is frequently found in small odontocetes, especially in the harbour porpoise.

PARASITOLOGICAL EXAMINATION

Fred Borgsteede

Introduction

It is well known that animals at the end of the food chain, such as small cetaceans, may be heavily parasitized by many species of endoparasites belonging to different phyla of the animal kingdom (Dailey and Stroud, 1978; Delyamure, 1954). In this contribution two topics will be discussed: where parasites can be found most often inside the cetacean body, and which dissection and sampling techniques may be used for parasitological investigations. This overview, with no intention of being comprehensive, is based on the common findings of parasites in small cetaceans of the North Sea. In other geographical regions other parasite species can be found, but the basic dissection and sampling techniques vary little, if at all.

Where can endoparasites be found in the cetacean body?

In principle, endoparasites may occur throughout the whole body of cetaceans and therefore all organs should be examined for their presence. Below, an overview of the most common parasites and their target organs is given.

Gastrointestinal tract
In the first section of the stomach, mainly nematodes belonging to the group of ascarids can be expected (Smith, 1989). Occasionally lungworms are found. How exactly these reach the stomach is unknown. Possibly they are swallowed after being coughed up. Stomach trematodes have been reported from other waters (Schryver *et al.*, 1967).

In the stomach wall of porpoises, particularly in the pyloric section, cystic cavities which contain the trematode *Pholeter gastrophilus* may be present. These cystic cavities are visible with the naked eye and vary from a small thickening to cysts with a circumference of 20 cm.

The intestine may contain cestodes belonging to the genus *Diphyllobothrium*, trematodes and acanthocephalans (for example: *Corynosoma* spp., *Bolbosoma* spp.). Cestodes and acanthocephalans are visible with the naked eye, trematodes under a low magnification microscope (x10 or higher).

Liver
The liver fluke *Campula oblonga* (Trematoda) is very common in the bile ducts, and occasionally found in the pancreatic ducts.

Lungs
Several nematode species parasitize the lungs (Arnold and Gaskin, 1975). In the trachea, bronchi, bronchioli and alveoli worms of the genera *Pseudalius, Torynurus, Stenurus* and *Halocercus* are common. Worms of the latter genus are frequently found in the lung parenchyma.

Circulatory system
Pseudalius inflexus may be found in the right side of the heart, pulmonary artery, and pulmonary blood vessels.

Cranial sinuses
The ventral sinuses of the head of porpoises (behind the eye, around the tympanic bullae, the pterygoid sinuses, etc.) are often invaded by the nematode *Stenurus minor*.

Other locations
Parasites may occasionally be found in the fat tissue, the blubber (especially around the mammary glands), the interface between blubber and muscle, the lactiferous canals, the abdominal wall, the kidneys and ureters. These are often nematodes belonging to the genus *Crassicauda* or plerocercoids of cestodes (e.g. *Phyllobothrium* spp.).

Which dissection and sampling techniques may be used for parasitological investigations?

General methods
1. The best fluid to fix and store material for histological examination of parasite related body reactions is 10% neutral-buffered formalin. Fixation and storage of helminths for parasitological examination is best in 70% alcohol plus 5% glycerin. The glycerin prevents worms from drying out, if the alcohol has evaporated. However, fixation in 4% formalin (1,6% formaldehyde) is also suitable.

2. Identification of worms is possible at different levels.
The first identification level is the group to which the worms belong: *nematodes, trematodes, cestodes* and *acanthocephalans*. Within these groups one may identify the genus and species (second level). Individual worms may be classified according to sex and stage of development (third level). An old, but useful book on parasites in cetaceans is the one of Delyamure (1955).

Stomach
*Minimal examination
Open the stomachs and look macroscopically for the presence of parasites. If present identify them to the group level.

*More extensive examination
Open the stomachs. Collect all visible worms in a graded beaker and read off the estimated volume of the parasites from the side of the beaker if there are many, or count them if there are few. Fix and store the parasites. Identify the worms to the genus or species level.

*Optimal examination
Open the stomachs and wash them carefully under running tap water. Collect all the washings in a bucket. Run the contents over a sieve with a mesh size of 0.2 mm or less. Collect the parasites from the sieve and fix them in 4% formalin. It may be necessary to take a subsample of the contents of the stomachs or the intestine if there is a large amount of material, e.g. one tenth. In that case the number of parasites counted needs to be multiplied by ten to estimate the total number. Any sample can be investigated for the presence of worms by use of a microscope (magnification x10 or higher). Store the collected worms.

Stomach wall of the pyloric section
*Minimal examination
Look macroscopically for the presence of cysts.

*Optimal examination
If cysts are present, measure the circumference of the thickening caused by the cysts. As it is very difficult to collect all worms, a worm count is not practical. However, to confirm the presence of *P. gastrophilus* collect and fix worms by cutting into the cysts and pressing them out.

Intestine
*Minimal examination
Open the intestine at regular intervals and look macroscopically for the presence of parasites. If present, classify them at the first level.

*More extensive examination
Open the whole intestine. Collect all visible worms. Estimate the volume or wet weight of the tapeworms. Fix and store the worms. Identify the worms to the genus or species level.

*Optimal examination
Open the whole intestine. Wash the opened intestine carefully under running water. Collect all washings in a bucket. Look carefully for parasites adhered to the intestinal wall (acanthocephalans!). Follow then the same procedures as given for the stomachs.

Liver

*Minimal examination
Look macroscopically for signs of presence of worms ("white spots", fibrotic bile ducts, calcifications). Cut some slices and see if parasites can be pressed out.

*Optimal examination
Cut the whole liver into slices of 1 cm thick. Wash and press the slices in a bucket filled with water. Remove the pressed-out slices. Sieve the contents of the bucket over a sieve with a mesh size of 0.3 mm. You may take a subsample or investigate all material. Count the worms and, if desired, classify them according to stage. Fix and store the worms as indicated above.
Liver flukes may also be found (rarely) in the pancreatic ducts or the pancreas. If so, the same procedures as described for the liver can be applied.

Lungs
*Minimal examination
Look macroscopically at the lung surface for the presence of parasites. Cysts of *Halocercus* or nodules as a reaction to the presence of *P. inflexus* may be seen on the outside. Cut the lungs open, starting with the trachea and continuing with the bronchi and main bronchioli. Look for parasites in these places.

*More extensive examination
Follow the same procedure as described above, but pick out all worms and estimate their total volume. One may distinguish between worm species. An easy distinction is made between "large" lungworms (*P. inflexus*) and the smaller ones (all other species).

*Optimal examination
Collect and count all worms that can be seen macroscopically. Cut the lungs in slices to see how heavy the infection with *Halocercus* spp. is. Investigation of the lungs is far from easy. Care is needed to remove *P. inflexus*, because the head of the worm is almost always curled up in a nodule deep in the lung tissue and the worm will break if pulled from the parts located in the trachea, bronchi or larger bronchioli. Entangled masses of *Torynurus* are easily removed from the main bronchioli, and may also be found coughed up in the upper airways. Fix and store the worms as described earlier and identify them according to species, sex and stage.

Circulatory system
*Minimal examination
Cut open the right side of the heart to look for *P. inflexus*. Also look in the major blood vessels of the lungs.

*Optimal examination
Investigate the circulatory system as indicated above. Collect all worms and count them. If good specimens are needed for a collection, those collected from the blood vessels are almost always undamaged. Fix and store in the usual way.

Cranial sinuses
*Minimal examination
Examine all locations where *S. minor* may occur, such as tympanic bullae, sinuses behind the eye, pterygoid sinuses, etc. Simply note if parasites are present or not.

*Optimal examination
Carefully examine all locations in the head where *S. minor* may occur. Collect all worms. Count and identify them. Look for differences in worm numbers at different locations in the head.

Other organs
*Minimal examination
Examine the other organs, especially the fat tissue, the blubber (especially around the mammary glands), the interface between blubber and muscle, the lactiferous canals, the abdominal wall the kidneys and ureters for the presence of parasites.

*More extensive examination
Same as above, but also collect the parasites for identification.

*Optimal examination
Same as above, but count the number of parasites at the different locations.

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Discussion

High parasite burdens are often found in the lungs of stranded harbour porpoises. However, one has to be careful of the significance one attributes to this finding, as by-caught animals may have heavy lungworm burdens as well. Mörner stated that the lungs of roe deer also have high parasite burdens without necessarily debilitating the host.

It is possible that parasitic infections in cetaceans facilitate secondary infections with bacteria or viruses, but there was no confirmed data available to demonstrate this link.

An important point of discussion was how to quantify the parasitic burden. Counting the individual parasites is the most accurate quantitative method, but is time consuming. A qualitative statement (e.g. "heavily infested") is quickly made, but has little value as it is so subjective. A compromise between these two extremes is to measure the volume of the parasites present. However, no generally satisfactory method was agreed upon.

The method, recommended by Borgsteede, of preserving parasites in alcohol with glycerol was briefly discussed. Other options are to store parasites in alcohol without glycerol, or in formalin. The disadvantages of formalin are that it is unpleasant to work with, that the maximum air concentrations

in working places are regulated by law in many countries, and that it may distort the shape of the parasites, making their identification more difficult. It was generally agreed that 70% alcohol alone is a suitable fixative if it can be reasonably assumed that the container will not allow evaporation of the liquid.

TOXICOLOGICAL EXAMINATION

Michael Walton

Introduction

There are many different toxic or potentially toxic chemicals in the marine environment and most of then arise from urban, industrial and agricultural discharges. Since it is obviously impractical to screen biological samples for the possible presence of all such compounds, some selection has to be made on which ones should be monitored. Those regarded as lower priority are the large number of water-soluble compounds which are soon dispersed or readily broken down and which tend to be present in very low concentrations in both sea-water and marine animal tissues. Those given high priority are compounds which are neither readily degraded nor metabolised and which tend to accumulate in animal tissues by up to several orders of magnitude over the concentrations found in sea-water. This latter effect becomes more noticeable as one moves up through the food chain and so it is of particular concern to marine mammals which are top predators. Among the compounds which bioaccumulate, those which cause most concern fall into two main groups, namely the heavy metals and the organochlorines which have well documented toxic effects (at least in terrestrial mammals).

The methodology to be employed in collecting and preserving samples depends on several factors, e.g. what pollutants are to be analyzed and the method of analysis. For specific details it is always advisable to consult the laboratory performing the analyses. This contribution will therefore concentrate on giving some general guidelines on these points.

It should also be noted that other organic pollutants such as polycyclic aromatic hydrocarbons, which do not fall into either of the two above categories, are also currently being investigated by some groups.

Organochlorines (OCs)

Some of these compounds are or have been widely used as pesticides and herbicides (e.g. DDT, dieldrin, HCB). Others such as polychlorinated biphenyl compounds (PCB's) are/were extensively used in many industrial products such as transformer oils, paints, plastics, hydraulic fluids, and fire-retardants. The highly toxic dioxins and dibenzo-furans are formed as industrial by-products. Although the use and manufacture of many OCs has been banned in the West during the last 10-20 years, there are still vast amounts still being used in old equipment, etc., which could potentially leak into the environment. Despite the ban there has not been a significant decline in PCB contamination found in marine mammal tissues over the past 15-20 years (Loganathan and Kannan, 1991).

In marine mammals, PCBs have been shown to have detrimental effects on the reproductive system and to lower levels of vitamin A and thyroxin (Brouwer *et al.*, 1989; Reijnders, 1988) They are also thought to lower the efficiency of the immune system as they do in terrestrial animals (Vos and Luster, 1989). Organochlorines are characterised by low metabolism and their high solubility in neutral lipids such as triglycerides.

Analysis

There are a number of technical problems in the analysis of OCs which make the procedure somewhat time-consuming and relatively expensive to perform. For example, there are theoretically 209 different PCBs (congeners) based on the number and configuration of Cl atoms and they differ both in their toxicity and persistence in the environment. Thus it is desirable to determine individual congeners rather than estimate the total PCB content. The most toxic congeners are thought to be the coplanar PCB's which are often present in relatively low concentrations and are usually assayed by a different procedure to the remaining congeners. Analysis generally involves extracting a lipid fraction, removing the lipids by a column chromatography followed by separation on a gas chromatograph and/or a Mass spectrometer. Problems, depending on the method used, can arise from interference by other compounds especially phthalate plasticisers. These interfering compounds can be easily taken up from certain plastics by fatty tissue.

Sampling

a Use a clean, sharp knife to cut out the sample (about 5-10 g although more is preferable) and avoid contact (at least until checks have been on made their suitability) with plastics, waxed containers, rubber seals, cardboard and cloth bags. The sample can be placed into a hexane-rinsed glass container with a teflon seal or a screw lid separated from the sample by aluminium foil. In a field situation or with large samples, wrapping the tissue in hexane-rinsed aluminium foil would be satisfactory.

b The sample should be kept cool and frozen as soon as possible and then stored frozen until analysis. It is of interest to determine pollutant burdens both on a wet weight basis and a lipid weight basis. Therefore repeated freeze-thaw cycles should be avoided as this will break down the integrity of the cells and lipid will flow out of the blubber mass and it will be consequently difficult to obtain reliable results on a wet weight basis.

c The tissue usually analyzed for OCs is blubber since it usually has the highest lipid and organochlorine contents. However, for a number of reasons it may not be the best indicator of pollutant burdens so in addition to blubber it would also be useful to collect samples of liver, kidney and muscle and maybe melon (for dolphins). It is important to obtain a representative sample, e.g. for blubber the whole layer from skin to muscle. In some species the structure and composition of the blubber varies in different parts of the body. Thus, until it has been shown for a given species that the pollutant has a uniform distribution in the total tissue mass, one should collect samples from a number of sites or from a standardised location in each animal. (See also general points.)

Heavy Metals

A number of heavy metals (e.g. lead, chromium, mercury, nickel, copper, zinc, cadmium) are often analyzed but perhaps there is most concern for lead, cadmium and mercury. Most metals are present as inorganic chelates or a cations but mercury is commonly found as organic methylmercury which is more toxic than the free form. Most heavy metals concentrate in the liver but this is not always the case; lead can accumulate more in bone and cadmium in the kidney. Lead poisoning can lead to severe encephalopathy and brain damage; mercury can damage the nervous system and kidneys; and cadmium can cause lung damage.

Analysis

They are generally analyzed by atomic absorption spectrometry which is often specific for each metal so the main concern in collecting samples is to avoid contamination from a number of sources (see general points).

Sampling

a A clean high quality knife should be used for cutting. Samples can be kept in polythene bags or acid-washed glass containers with teflon seals. They should be kept cool and frozen as soon as possible.

b The main samples to collect are liver, kidney and muscle, but a sample of bone would also be useful for lead. Again for comparative purposes one should be consistent in the sampling site, e.g. concerning the left or right liver lobe, kidney, muscle sampling site etc.

General Points and Summary

1 Collect samples in a "clean" environment. Avoid places where there may be atmospheric contamination, e.g. due to petrol and diesel fumes, paints, aerosols, smoke, cosmetics, talc, rust, and dust.

2 Use clean, good quality tools to cut and handle the tissues. Thus avoid rusty knives or knives with small fragments adhering to the blade after sharpening. Try and avoid contact with plastics for tissues destined for pollutant analysis.

3 The tissue should be stored in suitable containers which will not interfere with the subsequent analyses, frozen as soon as possible, and kept frozen until the time of analysis. If samples have to be sent through the post do it by the fastest means possible and insulate the container well.

4 Even when samples are analyzed in a suitable manner it is not always easy to assess the significance of the results obtained, especially from stranded animals. The levels of organochlorines may be influenced by factors such as age, sex, location, sampling site, nutritional state, health state, reproductive state, when collected, and how long after death the sample was collected. Therefore it is necessary to record as many of these details as possible when collecting the samples. (See Aguilar (1987) for a more detailed discussion of these points.)

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Discussion

A number of participants emphasized that it is necessary to collect samples for OC analysis from more tissues than just the blubber, to be able to learn more about the distribution and metabolism of OCs throughout the body. However, the high costs of analysis are often a limiting factor.

It was asked if it was necessary to wash the aluminium foil with hexane. Walton answered that as far as he knew unwashed aluminium foil had not been shown to affect the OC levels, but he advised the use of hexane-washed aluminium foil as an extra precaution.

PROPOSAL FOR A STANDARD PROTOCOL FOR BASIC POSTMORTEM EXAMINATION AND TISSUE SAMPLING

Thijs Kuiken and Manuel García Hartmann

A draft for a standard protocol for basic postmortem examination and tissue sampling of small cetaceans was presented to the workshop participants. This draft was based on existing protocols from a number of sources - UK marine mammal stranding and by-catch project, German cetacean stranding and by-catch project, CRC handbook of marine mammal medicine, California Marine Mammal Center, Charleston laboratory forensic manual, report of the international workshop on the harbour porpoise held at Cambridge in 1988 (ECS newsletter no. 6) - and on personal experience. The draft was discussed and comments and suggestions made by workshop participants were incorporated into the protocol and recording form, that can be found in appendices 3 and 4.

Discussion

Stranding information
The term "body condition", used in this protocol to indicate the state of decomposition of a carcass, may cause confusion because this term is also used to indicate how fat or thin a carcass is.

There was some discussion whether categories 3 and 4 of the condition code should be amalgamated, as it would be difficult for a lay person to differentiate between the two categories. Finally, it was decided to leave them as they were, as it was useful to know if a carcass was thought to be in category 3 or 4, and for the sake of standardisation with countries where these categories are already in use. It was noted that the abdominal cavity of a carcass in category 3 is often still closed, while in category 4 it is often open.

Body measurements and physical appearance
It was thought that it is often more useful to take a number of close-up photographs of a carcass, than an overview of the whole lateral side of a body. However, an overview photograph may help to record the state of decomposition, and any markings or external lesions.

Bacteriological examination
The difficulty of taking samples for the culture of anaerobic bacteria was noted: one needed to either use a speciál transport medium, or take a tissue block to be able to keep them viable.

Parasitological examination
It was mentioned that live parasites should be immersed in Berland's fluid (19 parts pure glacial acetic acid + 1 part concentrated formalin (= 40% formaldehyde solution)) for 1 minute for correct fixation. However, as live parasites are seldom found in cetacean carcasses, it was not thought necessary to include this in the basic protocol.

Toxicological examination
It was emphasised to take a full cross-section of kidney tissue, including cortex and medulla, for heavy metal analysis, because e.g. cadmium is found concentrated in the cortex.

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The following list, with no intention of being comprehensive, is partly based on the responses to a questionnaire sent to the national contact persons of the ECS. At the time of compilation there were replies from Belgium, Iceland, Germany, Sweden, Spain, France, Denmark, The Netherlands, Italy, Portugal, United Kingdom, and the Faroe Islands.

The area of interest of the scientists is indicated using the following code:

AGE	-Age determination using teeth
FOO	-Identification of food remains
GEN	-Molecular genetics
REP	-Reproduction
SKU	-Stock identification studies using skulls
PAT	-Pathology
VIR	-Virology
BAC	-Bacteriology
PAR	-Parasitology
TOX	-Toxicology

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APPENDIX 3:

STANDARD PROTOCOL FOR THE BASIC POSTMORTEM EXAMINATION AND TISSUE SAMPLING OF SMALL CETACEANS

CONTENTS

1. Collection and storage of samples
a) Condition code
b) Labelling of samples
c) Life history samples
d) Histopathological samples
e) Virological samples
f) Bacteriological samples
g) Parasitological samples
h) Toxicological samples

2. Gross postmortem examination and tissue sampling
a) External examination
b) Examination of abdominal organs (except gastro-intestinal tract, pancreas, and spleen)
c) Examination of organs of head, neck and thorax
d) Examination of the gastro-intestinal tract, pancreas and spleen

1. COLLECTION AND STORAGE OF SAMPLES

a) Condition code

The body condition, or state of decomposition of a body, can be described using the following categories:

1) **live** (becomes code 2 at death)
2) **extremely fresh** (as if just died, no bloating, meat is considered by most to be edible)
3) **moderate decomposition** (bloating, skin peeling, penis may be extended in males, organs still intact, excluding postmortem damage)
4) **advanced decomposition** (major bloating, skin peeling, penis extended in males, organs beyond recognition, bones exposed due to decomposition)
5) **indeterminate** (mummified carcass or skeletal remains, no organs present)

It depends on the body condition of a cetacean carcass which samples it is possible or useful to take. The following list provides a general guideline for each discipline (N.B. The clinical examination and sampling of live stranded cetaceans is not discussed in these guidelines):

Life history samples	2-5
Histopathological samples	2-3
Virological samples	2-3
Bacteriological samples	2-3
Parasitological samples	2-3
Toxicological samples	2-3

b) Labelling of samples

As the samples from each animal are to be examined by a number of people, often in different laboratories, it is very important that the samples are properly labelled, to ensure that it is possible for all people involved to trace a sample back to the tissue/organ and to the carcass it was taken from. This means that:
-each sample should have a label which is securely fastened to it;
-indelible ink or pencil should be used to write on the labels;
-the label should include at least the reference number of the carcass and the name of the sample.

c) Life history samples

Teeth (age determination)
Collection: At least 4 teeth should be collected from each individual. Preferably take the teeth from the middle of the lower jaw, as these are generally the straightest. From the harbour porpoise, teeth can easily be extracted by inserting a sharp knife or scalpel, firstly on one side of the gum in between the teeth and the connective tissue, and then on the other side. With a little pulling, an entire string of teeth can be removed. In species such as the bottlenose dolphin, the teeth can be loosened by prying in between the space between the tooth and the socket with a sharp pointed instrument. If this is not possible, a piece of mandible containing at least 4 teeth can be sawed off.

Storage: Loose teeth and jaw sections can be stored frozen. The temperature at which they are frozen is not critical. Alternatively, they can be fixed in 70% ethanol or 10% neutral-buffered formalin. They should not be stored dry as this may lead to cracking of the teeth.

Food remains (prey studies)
Storage: Food remains from the stomach can be stored frozen, or alternatively stored in 70% ethanol. The temperature at which they are frozen is not critical.

Skin (DNA studies)
Skin samples can be stored frozen at -20°C.

Gonads (reproduction studies)
The ovaries should be placed whole in 10% neutral-buffered formalin. Each testis should be weighed separately, without epididymis. Then the testes should be incised at 1 cm intervals before placing in 10% neutral-buffered formalin to allow the formalin to penetrate the tissue sufficiently. Use at least 10 times as much formalin as tissue when fixing. Once fixation has taken place, after about 24 hours, the samples can be stored in a smaller volume of 10% formalin.

If the testes are heavier than about 50 g each, place a cross-sectional slice about 1 cm thick from mid-way along the length in formalin after weighing the testes.
2
Skull (morphometrics studies)
The skull may be stored frozen. The temperature at which it is frozen is not critical.

d) Histopathological samples

Collection: In grossly normal organs, take a random sample of 1 cm^3 unless otherwise specified. Any lesion should be sampled with an adjacent piece of normal tissue. Use a sharp scalpel to take the sample, and try not to damage it (e.g. squeezing with a forceps) as this will make histopathological examination more difficult.

Fixation: 10% neutral-buffered formalin is a suitable fixative[1]. As a rule, formalin will only penetrate about 1 cm in any direction, so inject or cut samples larger than 2 cm in diameter to expose more tissue to the formalin. Also remember to try to have at least 10 times as much formalin as tissue when fixing. The tissues should remain in this fixative for at least 24 hours.

However, the brain should be placed in at least 20 times as much formalin as tissue when fixing it whole, and should remain in this fluid for at least a week. To allow faster fixation, a longitudinal incision can be made in the cerebrum to expose the lateral ventricles.

Storage: Once fixation has taken place, the samples may be stored in a smaller volume of 10% formalin.

e) Virological samples

In general, the choice of tissue samples for virological examination should be made in collaboration with a virology laboratory, taking into account (clinical and) pathological signs of stranded animal(s), and the state of decomposition of the carcass. The tissues for virological examination listed in these guidelines- lung, brain and kidney - are the minimum samples required for morbillivirus detection. Any lesions of suspected viral etiology should be sampled in the same way.

For virus isolation, samples of 2x2x2 cm from the relevant organs should be collected aseptically and placed in sterile containers. If they are sent to a virological laboratory within 24 hours, they should be kept at 0-4°C, otherwise they should be frozen at -70°C until analysis can take place. Frozen samples can be suitably transported in solid CO_2 (dry ice).

To carry out serological examination for viral and other diseases, a blood sample of at least 1 ml should be collected to obtain serum[2]. Until analysis, serum can be stored frozen at -20°C. Even if the serum is haemolytic, it is still of value.

f) Bacteriological samples

As a minimum, bacteriological examination should be carried out on samples of lung tissue, heart blood, and any lesions of suspected bacterial etiology.

Collection: In general, one should try to work as aseptically as possible. Samples for bacteriological examination can be taken by using swabs or tissue blocks. Each of these methods has its own advantages and disadvantages, and the choice depends on the preference of the person carrying out the examination, and of the relevant bacteriology laboratory.

To take a block of tissue, one should cut out a block approximately 4x4x4 cm using a sterile scalpel and forceps, and place this in a sterile container. Samples should be kept at 0-4°C until processing, which should be carried out as soon as possible.

To take a swab, one should disinfect the surface of the organ, for example with 70% alcohol, incise it with a sterile scalpel, and place a sterile swab in the incision. The swab then can be inserted in a

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[1] 10% neutral-buffered formalin can be made as follows: dissolve 3.31 g $NaH_2PO_4.H_2O$ and 33.77 g $Na_2HPO_4.7H_2O$ in 1 l distilled water. To 1 part of concentrated formalin (= 40% formaldehyde solution) add 9 parts of this buffer.

[2] To separate serum from blood cells, the blood sample should be centrifuged at no more than 2200 revolutions per minute for 10 minutes and the supernatant serum collected.

sterile tube with transport medium. Sterile swabs and containers with transport medium are available commercially as packages. A suitable choice is a package for aerobes and anaerobes, containing for instance charcoal transport medium. The swabs should be kept at 0-4°C until processing.

To take a heart blood sample, one should disinfect the surface of the right ventricle, for example with 70% alcohol, and take a blood sample from the lumen using a sterile syringe and needle. One can also use a sterile pipette. In both cases, the blood sample should be stored in a sterile container at 0-4°C until further processing.

Processing: Smears from tissue samples should be fixed, and stained with Gram's method. Smears of lung samples and of samples from lesions of suspected mycobacterial etiology should also be stained using Ziehl-Nielsen's method.

Samples should be inoculated onto for example Columbia Blood Agar and incubated for 5 days at 37°C aerobically and anaerobically, except for the lung sample, which need only be incubated aerobically.

g) Parasitological samples

As many parasites as possible should be sampled, depending on their numbers and the difficulty of collecting, and preserved in 70% ethanol. The total number of parasites should be estimated. If parasites are associated with lesions, these should be sampled, if possible with parasites attached, and fixed in 10% neutral-buffered formalin for histopathological examination.

h) Toxicological samples

Collection: Samples of blubber, muscle, liver, and kidney should be taken for organochlorine analysis. These samples should only come into contact with stainless steel, aluminium, glass or teflon, and can be stored in contaminant-free hexane-washed aluminium foil.

If the animal was lactating, a milk sample should be collected for organochlorine analysis. This can be stored in a contaminant-free hexane-washed glass container, with contaminant-free hexane-washed aluminium foil to keep the sample from contacting the (plastic) cap of the container.

If there is a foetus present too small for full postmortem examination, the whole foetus and its placenta can be wrapped in contaminant-free hexane-washed aluminium foil for organochlorine analysis.

Samples of muscle, liver, and kidney should be sampled for heavy metal analysis. These samples should not come into contact with any metals other than stainless steel, and can be stored in plastic.

The minimal size of the samples for heavy metal and organochlorine analysis is 10 g (10 ml for milk), and the samples should be weighed if they are to be frozen, to be able to compensate in the results for dehydration during storage.

Storage: If samples are analyzed directly after collection, they do not need to be cooled. Otherwise, they should be frozen at -20°C until analysis and not thawed during storage, otherwise the tissue will break down.

2. GROSS POSTMORTEM EXAMINATION AND TISSUE SAMPLING

This protocol has been written under the assumption that there is sufficient time and that the circumstances are suitable to carry it out. If this is not the case, one should always attempt to collect the following data (in accordance with priority category 1 of the ECS harbour porpoise workshop, Cambridge 1988, ECS Newsletter no. 6): species, sex, location and date found, body length, teeth, gonads.

All structures must be examined visually and by palpation, making incisions into the organs. A full post mortem record must be kept. As an example, the standard "recording form for cetacean postmortem examination" is included below.

Lesions in any organs should be described and sampled. The description should include the size, location, colour, texture, shape, and the nature of the transition from normal to abnormal tissue. Any unusual lesions should be photographed. Photogra phs should include a ruler or similar object to indicate the size of the lesion. According to the suspected etiology of the lesion, samples should be collected for bacteriological examination (especially if the lesion is of a purulent nature), for virological examination, and/or for parasitological examination. In all cases, a sample of the lesion should be preserved for histopathological examination (see respective sections in Chapter 1).

Any parasites found, regardless whether they are associated with pathological lesions or not, should be collected for identification (see "Parasitological samples"). Some predilection sites for parasites are indicated in the text.

The postmortem examination need not necessarily take place in the order described below. However, samples for bacteriological and virological examination need to be taken as early as possible. Also, care should be taken to prevent cross-contamination with enteric micro-organisms. For this reason, the examination of the gastro-intestinal tract should be left until last, or carried out completely separately.

a) External examination

body measure-ments

Photographs should be taken of the lateral views of the whole body, from both sides.

Estimate the body condition, or state of decomposition of the carcass, using the categories of the condition code (see "Condition code").

Weigh the animal in kg. If this is not possible, the body weight can be estimated from the heart weight (see below).

Measure the length in cm by placing the carcass on its belly, holding a measuring tape or ruler next to the carcass in a straight line parallel to the longitudinal body axis and measuring the distance between the notch in the tail flukes and the tip of the upper jaw.

nutritional state Describe the nutritional state of the carcass. A thin blubber layer and muscle atrophy are signs of a poor nutritional state. Visible signs of atrophy of the musculature are a hollow appearance behind the skull and lateral to the dorsal fin, and protrusion of the lateral processes of the lumbar vertebrae after taking off the blubber.

skin Examine the animal for external lesions and sample these accordingly. Examine the skin carefully for any ectoparasites. These are most likely to be found in or near the body openings and next to the fins and flukes. Take a 4 cm^2 piece of skin down to the blubber for DNA-studies (see "Life history samples").

body orifices Examine the mouth (including teeth, tongue, and buccal cavity), eyes, ear openings, blow-hole, anus, genital slit and mammary slits for lesions and any discharge.

milk Massage the skin in the area cranial to the mammary slits in a caudal direction to express any fluid present in the mammary glands. If fluid can be pressed out, take a sample for organochlorine analysis (see "Toxicological samples"). Note the volume, colour, and consistency of the fluid.

blubber Cut a cross-sectional strip of blubber a few cm wide and a few cm long at the level of the caudal insertion of the dorsal fin. Make sure to cut at right-angles to the surface of the skin. Measure the thickness of the blubber strip in mm just beside the dorsal mid-line. (Using this method, the tension of the blubber tissue is relieved before measuring.)

From this blubber strip, take a cross-sectional sample of blubber for organochlorine analysis (see "Toxicological samples"). It is important to take the blubber sample of the whole layer, from the skin to the muscle.

muscle Take 2 muscle samples: one for organochlorine analysis, one for heavy metal analysis (see "Toxicological samples"). The muscle samples should be taken at the same location as and directly below the blubber sample, at the level of the caudal insertion of the dorsal fin.

With the animal on its right side make a mid-line ventral incision from the symphysis of the mandible to a short distance posterior of the anus, circumventing the genital slit and anus. From the posterior end of the ventral incision make a second one almost to the dorsal mid-line. Take the skin and blubber off the uppermost side. Any parasites in the blubber should be noted and collected. These may occur as white cysts less than 1 cm in diameter, often in the ano-genital region or the dorsal aspect of the chest wall.

mammary gland In females, cut into the mammary gland and examine it for the presence of fluid, parasites, and pathological changes.

subcutaneous tissue Examine the subcutaneous tissue for the presence of bruises and parasites.

b) Examination of abdominal organs (except gastro-intestinal tract, pancreas, and spleen)

Remove the left abdominal wall, freeing the testis or ovary and uterus. Any parasites in the abdominal wall (for instance cysts under the peritoneum) should be placed in ethanol. Remove the left thoracic wall, for example with bone shears.

virol. and bacteriol. samples

Before handling the internal organs, take a sample of lung and kidney tissue for virological examination (see "Virological samples"). Also take a sample of lung tissue, preferably from the cranio-ventral part of the left lung, and heart blood, preferably from the right ventricle, for bacteriological examination (see "Bacteriological samples").

Sever the intestine close to the anus and the oesophagus close to the diaphragm. Working forward along the dorsal aspect of the abdominal cavity, remove the stomach, intestines, pancreas, spleen and mesenteric lymph node, attached to each other, from the carcass. Leave the examination of the gastro-intestinal tract to the end of the postmortem examination, or carry it out completely separately, to prevent cross-contamination of other tissues with enteric micro-organisms.

urinary bladder

Open and examine the bladder in situ, noting the contents, if any.

female reproduct. tract

In females remove the entire reproductive tract, open the vagina and uterus, note any corpora lutea or albicantia or follicles on each ovary and then keep the ovaries for reproduction studies (see "Life history samples").

foetus

If a foetus is present of sufficient size to examine the individual organs, a postmortem examination and tissue sampling of the foetus can take place in the same way as for cetaceans after birth. If it is too small for a full postmortem examination, the whole foetus and its placenta can be kept for organochlorine analysis (see "Toxicological samples").

male reproduct. tract

In males remove the testes, incise them at 1 cm intervals, examine the cut surface and keep the testes for reproductive studies (see "Life history samples"). Examine the penis and preputium.

adrenal glands

Remove and examine the adrenal glands. Collect a cross-sectional slice of about 1 cm thick from halfway the length of the left adrenal gland for histopathological examination.

kidneys

Remove the kidneys from the body cavity. Incise both kidneys longitudinally, and if possible, strip the capsule. Then, take 2 samples: one for organochlorine analysis, one for heavy metal analysis (see "Toxicological samples"). These samples should be cross-sectional and include both medullary and cortical tissue. Preserve 1 cm^3 from a kidney for histopathological examination.

liver Remove the liver, examine both surfaces and make multiple incisions into the substance. Examine the bile ducts for parasites. Then, take 2 samples: one for heavy metal analysis, one for organochlorine analysis (see "Toxicological samples"). These samples should include approximately equal amounts of tissue from the edge of the left lobe, the edge of the right lobe, and the hilus of the liver. Collect 1 cm^3 of liver tissue for histopathological examination.

c) Examination of organs of head, neck and thorax

thyroid gland Carefully remove the superficial muscles overlying the trachea and larynx, so exposing the thyroid gland. Examine this tissue.

Incise along the internal aspects of both mandibles and free the tongue. Once the tongue is free pull it backwards and cut the hyoid bones close to the skull.

Free the larynx from the sphincter muscle holding it in place and pulling the tongue backwards incise along the neck to free the trachea and oesophagus. Then, incising dorsally and ventrally in the thoracic cavity, free the heart and lungs. Note any attachments of the lungs to the thoracic walls. This procedure should give you the tongue, larynx, trachea, oesophagus, thymus, heart and lungs all still fastened together.

tongue Examine the surface of the tongue.

oesophagus Open the oesophagus longitudinally and check for lesions or parasites.

respiratory tract Open the larynx, trachea and major bronchi longitudinally. Make multiple incisions into the substance of both lungs. Any parasites should be collected. Two pieces of lung (about 1 cm^3) from the hilus and periphery of the left lung should be taken for histopathological examination. The samples should include part of the major bronchial tree. Open all major branches of the pulmonary veins and examine for the presence of parasites. Examine the bronchial and so-called "pulmonary associated" lymph nodes. The latter can be found about halfway the ventral edges of each lung. Cut a 1 cm thick cross-sectional slice from halfway the length of the left pulmonary associated lymph node for histopathological examination.

thymus Examine the thymus, if present.

heart Collect any blood present in the heart lumen (or elsewhere), to obtain serum for serological examination (see "Virological samples").

Separate the heart from the lungs by cutting through the major blood vessels where they enter the heart. Open the left and right ventricles and atria for examination and collect any parasites found. Cut a 1 cm thick slice of heart tissue, to include a piece of the wall of the left ventricle and of the atrioventricular septum, for histopathological examination.

Open the atria and ventricles of the heart. Any parasites should be collected.

ear sinuses nasal sacs Examine the ear sinuses around the tympanic bullae, and the nasal sacs. By using a forceps collect any parasites present.

teeth If possible, remove at least 4 teeth from the middle of the lower jaw for aging (see "Life history samples").

brain In freshly dead carcasses (condition code 2), open the skull, and examine the brain.

The skull can be opened by making a vertical cut parallel and about 2 cm posterior to the transverse dorsal ridge which is clearly visible and palpable on top of the skull. The second cut should be made in the horizontal plane, through the occipital condyles, making sure to leave the posterior portion of the condyles on the skull, so that the condylo-basal length can still be measured. Both cuts should be extended until they meet each other. The separated piece of skull can then be prised loose using a chisel or flat bladed screwdriver, and the brain can be removed.

Take a sample of brain for virological examination (see "Virological samples"). Place the whole brain in formalin (see "Histopathological samples"). When it is fixed, make multiple slices into the tissue to look for pathological lesions, including the presence of parasites. Take 1 cm^3 samples of the cortex, midbrain, cerebellum, and medulla, for histopathological examination.

skull In more decomposed carcasses, leave the skull intact. Both opened and completely intact skulls should be kept for morphometrics studies (see "Life history samples").

d) Examination of the gastro-intestinal tract, pancreas and spleen

spleen Examine the spleen and collect a piece (about 1 cm^3), including a section of capsule, for histopathological examination. One often finds smaller accessory spleens near to the main spleen.

pancreas Examine the pancreas. Look for parasites, particularly in the pancreatic ducts. Take a 1 cm^3 piece of pancreas tissue for histopathological examination.

mesenteric lymph node Examine the mesenteric lymph node and remove a 1 cm thick cross-sectional slice from halfway its length for histopathological examination.

Examination of the gastro-intestinal tract is usually left until last, or carried out separately, to prevent cross-contamination of other organs with enteric micro-organisms.

stomach Open the cardiac section of the stomach. Remove fish bones, otoliths and all other food remains for prey studies (see "Life history samples"). Any parasites should be collected. Describe any lesions, including the distribution and size of any ulcers.

Open the fundic and pyloric sections of the stomach. Any food material and parasites should be treated as for the cardiac section. Any nodules in the walls of the fundic and pyloric sections should be noted and, if they are found, attempts should be made to express the contents. Any parasites found in the contents should be collected.

intestine Open the entire length of the intestine. Any parasites should be collected.

APPENDIX 4:

RECORDING FORM FOR CETACEAN POSTMORTEM EXAMINATION

IDENTIFICATION NUMBER:

OTHER IDENTIFICATION NUMBERS IN USE:

1. STRANDING INFORMATION

SPECIES:

SEX:

LOCATION FOUND:

DATE FOUND (day/month/year):

FOUND BY (name, address):

TOTAL # OF ANIMALS OF THIS SPECIES FOUND AT SAME LOCATION ON SAME DATE:

STRANDING CIRCUMSTANCES:

2. BODY MEASUREMENTS AND PHYSICAL APPEARANCE

BODY CONDITION USING CONDITION CODE:

Condition code:
1) **live** (becomes code 2 at death)
2) **extremely fresh** (as if just died, no bloating, meat is considered by most to be edible)
3) **moderate decomposition** (bloating, skin peeling, penis may be extended in males, organs still intact, except for postmortem damage)
4) **advanced decomposition** (major bloating, skin peeling, penis extended in males, organs beyond recognition, bones exposed due to decomposition)
5) **indeterminate** (mummified carcass or skeletal remains, no organs present)

PHOTOGRAPHS TAKEN? (lateral views of whole body from both sides)
YES / NO

BODY WEIGHT (in kg):

TOTAL LENGTH (straight line from tip of upper jaw to notch in tail flukes, in cm):

BLUBBER DEPTH (taken at the level of the caudal insertion of the dorsal fin, in mm):
DORSAL MID-LINE:

3. GROSS PATHOLOGICAL EXAMINATION

The following list is meant only as an aid to note which organs/parts have not been examined, show no abnormalities, or are abnormal.

Encircle the appropriate category:
NE = not examined
NAD = nothing abnormal detected
A = abnormal (describe fully below)

EXTERNAL EXAMINATION

NE NAD A -body orifices (mouth, eyes, ear openings, blowhole, anus, genital slit, mammary slits)
NE NAD A -dorsal and pectoral fins, tail flukes

INTEGUMENT

NE NAD A -epidermis
NE NAD A -blubber
NE NAD A -subcutaneous tissue
NE NAD A -mammary glands

MUSCULOSKELETAL SYSTEM

NE NAD A -skull
NE NAD A -other bones
NE NAD A -back muscle mass
NE NAD A -other muscles

NERVOUS SYSTEM

NE NAD A -brain
NE NAD A -spinal cord
NE NAD A -peripheral nerves

CARDIOVASCULAR SYSTEM

NE NAD A -pericardial sac
NE NAD A -myocardium
NE NAD A -valves
NE NAD A -arteries, veins

RESPIRATORY SYSTEM

NE NAD A -nasal cavity
NE NAD A -sinuses
NE NAD A -trachea, bronchi
NE NAD A -lungs
NE NAD A -pleura/pleural cavity

ALIMENTARY SYSTEM

NE NAD A -mouth
NE NAD A -oesophagus
NE NAD A -cardiac section stomach
NE NAD A -fundic section stomach
NE NAD A -pyloric section stomach
NE NAD A -duodenum/small intestine
NE NAD A -large intestine
NE NAD A -anus
NE NAD A -liver
NE NAD A -pancreas
NE NAD A -peritoneum/peritoneal cavity

UROGENITAL SYSTEM

NE NAD A -kidneys
NE NAD A -ureters
NE NAD A -urinary bladder
NE NAD A -urethra
NE NAD A -ovaries/testes
NE NAD A -uterus
NE NAD A -vagina/penis
NE NAD A -vulva/preputium

LYMPHATIC AND ENDOCRINE SYSTEMS

NE NAD A -adrenal glands
NE NAD A -thyroid gland
NE NAD A -spleen
NE NAD A -thymus
NE NAD A -lymph nodes

DESCRIPTION OF ABNORMALITIES GROSS PATHOLOGICAL EXAMINATION (add extra pages if necessary):

4. PRELIMINARY DIAGNOSIS OF GROSS PATHOLOGICAL EXAMINATION (in order of importance):

5. CHECKLIST OF STANDARD SAMPLES

In each square, enter a ✓ (= sample taken) or a X (= sample not taken or not present).

Record any extra samples taken in section 6.

ORGAN SYSTEM		SAMPLES FOR: histopathology (formalin, room temp.)	virology (sterile, 0-4°C or -70°C)	bacteriology (sterile, 0-4°C)	parasitology (ethanol, room temp.)	organochlorines (aluminium foil, -20°C)	heavy metals (plastic, -20°C)	life history (freeze)	life history (formalin, room temp.)	Comments
Integument	-skin				■			■		
	-blubber				■	■				
	-milk					*				*glass container
Musculoskeletal	-skull							■		
	-ear sinuses				■					
	-skeletal muscle					■	■			
Nervous	-brain	■	■		■					
Cardiovascular	-heart	1		2	2 3					1:muscle 2:blood 3:lumen
	-serum		*							* -20°C
Respiratory	-nasal sacs				■					
	-trachea				■					
	-bronchi				■					
	-lung	■	■	■	■					
	-pulmonary veins				■					
	-pulmonary ass. ln.	■								
Alimentary	-teeth							■		
	-stomach				■			*		*food remains
	-intestine				■					
	-mesenteric ln.	■								
	-liver	■			■	■	■			
	-pancreas	■			■					
Urogenital	-kidney	■	■			■	■			
	-gonads								■	
	-foetus					*				*only if too small
Lymphoid/endocr.	-adrenal glands	■								for postmortem
	-spleen	■								

20°C)

6. LIST OF EXTRA SAMPLES

Record any extra samples taken in the appropriate section below.

Extra samples of lesions taken for histological examination:

Extra samples of lesions taken for virological examination:

Extra samples of lesions taken for bacteriological examination:

Extra samples of lesions taken for parasitological examination:

Other extra samples taken: