STANDARDISED PROTOCOLS FOR THE COLLECTION OF BIOLOGICAL SAMPLES FROM STRANDED CETACEANS

These protocols will enable those authorised personnel responding to stranding events to undertake sampling in a manner that is consistent throughout Australia. They are designed to provide samples in the best possible condition for analysis by those scientists working in sometimes specialised areas.

Standardised methods and a prioritised order for collecting biological samples from stranded cetaceans will maximise the opportunity presented from often otherwise distressing and unsuccessful events involving whales and dolphins.

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These protocols have been developed by Damien P Higgins and Michael J Noad of Uniquest Pty Ltd. and the Australian Government Department of the Environment and Heritage through funding provided by the Natural Heritage Trust.

Their development follows consultation with a wide range of stakeholders and are designed to provide a national standard for the collection of samples from stranded cetaceans. It is hoped that the protocols will assist in productive, coordinated research across a broad spectrum of scientific investigation, and make maximum use of events where animals are not able to be returned to the sea alive.

Note: The collection of samples from cetaceans should only be undertaken by authorised personnel. Permits are required from state, territory or Federal government agencies prior to collection of samples of any kind from a live or dead cetacean and in most instances from state governments for movement of samples between states and into and out of Australia from the Australian Government Department of the Environment and Heritage.

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1.1 Rationale

Where live stranded animals are able to be assisted, the initial priority is to get them back into the water. Where this is not possible, biological sampling may be the only beneficial outcome.
The purpose of this document is to provide a national standard for biological sample collection from stranded cetaceans, to enable more productive, coordinated research to be carried out. It is restricted to sample collection and is not intended to provide any guidance in the areas of logistics, operations, triage and management of stranded animals or methods of treatment or euthanasia. **Workers are advised to refer to the response plans and operating procedures and policies of their respective state government.** While the protocols are consistent with best practice health and safety methods, **workers should abide by the health and safety requirements of their state government**.

1.1.1 Reasoning behind sample collection

In May 1996, the Biodiversity group of Environment Australia, now DEH, convened a workshop at Jervis Bay NSW on scientific and veterinary activities at cetacean strandings, in agreement with recommendations in the Action Plan for Australian Cetaceans (Bannister et al. 1996). The workshop reviewed concerns over:

- the high proportion of strandings for which little or no quantitative or biological information was available;
- the need to improve the basis for diagnostic and prognostic techniques in the field; and
- the need to assess the effectiveness of activities at strandings as reflected in long term survival following return of animals to the sea.

It was agreed that although components of these were being addressed already through more detailed response plans developed and implemented by some State and Territory agencies, there would be considerable benefit to a national approach.

The protocols have been developed to identify and seek the collection of samples that could be used to investigate the following, more detailed questions:

- Why do whales strand? (Basic signalment/ life history and stranding data, diagnostic pathology and acoustic trauma studies)
- Assessment/ improvement of management of live stranded cetaceans- (correlation of clinical examination and clinical pathology data with necropsy findings and post-release tracking information)
- Taxonomic/ anatomical material of rare species
- Population biology (taxonomic, population and social- structure genetics and basic morphologic signalment)
- Forage analysis especially with respect to fisheries
- Banking of samples for long-term disease and toxicological monitoring and for retrospective studies of emergent diseases
- More specific cetacean biology/ physiology/ health and disease projects (of variable priority)

It is expected that requirements will change over time with museum and tissue/ serum banking requirements remaining stable but university requirements changing more often and any successful system will need to accommodate this. For example, priority to take heads suitable for auditory analysis through MRI and CT scans has recently become a priority in some situations.

1.1.2 Comments on the use of protocols

No single protocol can be applicable to all situations. The detailed protocols are intentionally comprehensive to encourage the collection of full suites of samples and data under ideal conditions but a range of compatible tiered protocols are also provided to allow the level of sampling to be chosen at the discretion of the operator. In all cases the aim is to minimise the amount of time spent handling samples and fixatives on the beach (a potentially dirty, relatively uncontrolled, hazardous and complex environment), transferring crude samples to a more easily controlled environment for trimming, cleaning up, aliquoting and preservation.

1.2 Requirements

The collection of biological samples from stranded cetaceans is not a mandatory requirement and may only be carried out by those persons who already hold the relevant state or Commonwealth permits to do so. Authorised personnel are encouraged to prioritise sampling to stranding events where it is logistically possible and where the data obtained will be of greatest value.

1.2.1 State Responsibilities

States are responsible for stranded cetaceans on their territory. The Commonwealth is responsible for stranded animals on Commonwealth land. In practice therefore, the Commonwealth has few strandings, as most of the coastline is under state jurisdiction. The states issue permits for people to take samples from stranded animals and to move samples between states. States may also require sample collection from live animals to be approved by an ethics committee, and specific authorisations for special areas.

1.2.2 Commonwealth Responsibilities

The Commonwealth is responsible for controlling movement of samples into and out of Australia. DEH is able to issue permits under the ***Environment Protection and Biodiversity Conservation Act 1999*** for export and import which also meet obligations under the *Convention on International Trade in Endangered Species of Wild Fauna and Flora* (CITES).

1.3 Equipment and training

Protocols include equipment lists. Workers are encouraged to contact their state organisation for further information on collection or processing methods, prioritising sample collection or obtaining specialised equipment. Each state governing body has operating procedures and many regions have a register of local contacts for expertise and equipment.

1.4 Safety and Public health

Although there are few known zoonoses associated with cetaceans, it is likely that some zoonotic diseases remain unknown and therefore are likely to be difficult to diagnose and treat if contracted.

Of the known zoonoses, *Erysipelothrix rhusiopathiae* is the best known and is a frequent cause of "seal finger" or erysipeloid if humans are bitten. It is very common in marine environment on all marine mammals and fish and has been diagnosed in cetaceans on many occasions (Dunn et al 2001). Risk of entry is via breaks in the skin. (Duignan). Mycoplasmas and Vibrio (Dunn et al 2001) are also known to be transmissible from cetaceans to humans.

In addition the following organisms of marine mammals have potential to infect humans (Duignan 2000a, Duignan 2000b):

- Salmonellas (seals)
- Brucella (seals UK)

- Mycobacteria (seals) Has not been reported in cetaceans but transmission has occurred from seals to humans.
- M. marinarum in cetaceans (Dunn et al 2001)
- Influenza A (seals) Harbour seals- conjunctivitis in humans
- Seal Pox (parapox) Northern hemisphere- skin lesions
- Calicivirus Broad host range and potential for mutation- vesicular skin disease
- Rhabdoviruses (incl Rabies) Nth hemisphere cetaceans and seals
- Protozoa - Giardia, Cryptosporidium, broad host range cause gastrointestinal disease.
- Unknown

Safety precautions pertaining to collection of samples generally consist of basic hygiene and safety around sharp objects, but researchers and others operating at a stranding site should be aware of their state OH&S requirements. Basic conditions require:

- Adequate drinking water, and protection from sun, wind, cold, rain
- Good hygiene
- Double glove and apply barrier cream to skin
- Overalls
- Face mask and goggles if near blowhole or if tuberculosis is suspected
- Boot chains/ instep crampons for larger animals (to avoid slip accidents)
- Cover sharp bone fragments
- Chain mail gloves (optional- must be of good fit)
- Knife sharpeners- stones and steels
- Sharps containers and knife blocks
- Disinfect all cuts with iodine or alcohol 70%
- Disinfect all equipment after use with broad-spectrum disinfectant.
- Safety data sheets should accompany all chemicals eg fixatives, preservatives.

1.5 Clean up

After the stranding

- Clean up and disinfect all the gear, make sure that all the knives, the blubber hooks and the vernier callipers are washed in warm soapy water and the tape measures are wiped down and cleared of sand –spray those instruments that need it with WD-40 – this will stop them from rusting.
- Replenish all the kits with gear that has been used up e.g. sample bags, sample jars, gloves, 70% alcohol etc.
- Collate all the data collected and copy all data sheets. Make sure all sections are completed and all data collated.

Contact sample recipients to inform them of all samples collected and commence preliminary negotiations for the inclusion of any of the samples collected into his collections.

1.6 People Management, Media and Public Sensitivities.

Management of the many people likely to be involved in stranding events are covered in the operating procedures of each state, usually through an Incident Control System (ICS). Those people collecting samples, like the media and general public, also come under the control of the ICS manager.

Whale strandings are emotive events. The sampling and necropsy of deceased cetaceans may appear distasteful to some people – particularly to those who may have tried to save them. Discretion is required and in some instances, the ICS manager may find it useful to erect shields around animals being sampled.

1.7 Shipping of samples

See 5 below. (Processing of Samples).
Permits to move samples interstate or export from Australian (and import to) are required for all cetacean samples. Please contact the originating State Government agency and the Commonwealth Government (DEH) for guidance on what is required.

1.8 Protocol Forms

Protocols are form-based to facilitate their use on the beach (see Appendix 1). The protocols are tiered, and a guide is provided to assist personnel in choosing the tiers that are appropriate to their level of resources, expertise, and time (see Section 2). Each of the forms contains instructions on sample collection and preservation, and necessary equipment. It is important to map the initial characteristics of the stranding event through GPS or sketches of the position of the whales, high and low tide marks and times and other features that will assist with planning.
For those with time and resources to perform detailed sampling, or who desire more detailed instruction, or who are confronted with a mass stranding, more detailed protocols are provided in Sections 5 and 6.

1.8.1 P: primary stranding event data - all cetaceans (see Section 3)

The aim of this protocol is to:

- Provide instruction to those vets or people responsible for triage, who are not experienced in examination of cetaceans,
- Standardize the collection of clinical data and samples to compare with survival/ mortality data to assist the development of prognostic indicators.

The clinical assessment section of this protocol was presented at the Jervis Bay Workshop (Needham 1996).
For those who don't desire the level of instruction in this protocol, the P form is provided with the form-based protocols in Appendix 1.

1.8.2 T1: basic individual data and sampling- all animals and single dead cetaceans (see Section 4)

This protocol draws on the strong points of several existing protocols: In particular, collection of skeletal, basic biology and life history data and material is based on the protocol of the SA Museum (Kemper Unpublished) and the necropsy technique draws heavily on the guide by (Duignan 2000b). Being for a single animal it is the most comprehensive. It is best used in conjunction with diagrams by Geraci and Lounsbury, 1993 and Kemper, unpublished. Interpretation and description of observations during dissection is often difficult for the inexperienced, therefore it is recommended that photos be taken where possible and sent to experienced people or compared with photos of normal and diseased tissues placed on the website.
The protocol is designed for the best- case scenario, where time is not limited. Where time or resources are limited or where experienced personnel don't need the level of detail provided in this protocol, form-based protocols T1- T4 are recommended (Appendix 1). It is expected that the advice of those experienced in the field will continue to shape the protocol further.

1.8.3 T2-T4: individual data and sampling of increasing complexity - dead mass- stranded cetaceans (see Section 5).

The original protocol developed by Deborah Thiele (Thiele 1996) and modified by Karen Evans which is the basis for Section 5 is no longer in use in Tasmania. A new protocol for Tas DPIWE requires a modified ranking and tiered structure for mass standings.

1.8.4 Cl: clinical data and sampling - Sample preparation and preservation- common to all scenarios (see Section 5, p54).

This protocol is to be carried out at the closest suitable facility that is available (this may range from a tent off the beach to a local veterinary surgery or university). Ideally, a facility in each region could be identified and a kit of fixatives and disposables kept there. On occasion the suite of samples collected may be limited by the ability to get them to adequate processing facilities (centrifuges, refrigeration, fixatives) before they deteriorate. **Therefore, to avoid unnecessary work, this protocol should be read before commencing sample collection**. A summarized version of this protocol (CI) is provided with the form-based protocols in Appendix 1.

1.8.5 HI: Human interference data

For use where it is suspected that there has been some human interference, either before or after the stranding event.

1.8.6 SP: sample preservation, shipping, sample tracking forms

Additional information on research goals and best practice preservation of samples (Section 5). This document is a first step in providing workers with some additional information on sampling and sample preservation. Some alternatives to methods in the protocols are included as is some basic information on the reasons for collecting samples using the methods prescribed.

1.9 Prioritising sampling from carcasses at different stages of decomposition

Definitions (Geraci and Lounsbury 1993).

Table 1.1 Samples to be collected from carcasses at various stages of decomposition.

Sample	1	2	3	4	5
Clinical data for triage, treatment and later study	+				
Measurements and morphological description	+	+	+	**?**	
Photos/ description	+	+	+	+	+
Skin in EtOH or saturated salt with or without DMSO	+	+	+	+	**?**
Biopsies in 10% buffered formalin	+	+	+		
Blood in EDTA, Fl Ox, LiH and plain tubes - preferably at least twice during event	+				
Swabs of affected sites in transport media and frozen	+	+			
Chilled **affected tissues** (2cm cube in sterile container)		+	+		
Chilled non- contaminated **blubber, muscle, liver, kidney** (for virology, toxicology, fatty acid, stable isotope and hormone analysis)		+	+		
Blood at time of euthanasia in EDTA, Fl Ox, LiH and plain		+	+		

tubes or **Heart blood** in plain tubes					
Gross **pathology description**		+	+	**?**	
All organs and edges of lesions < 1cm thick slices in 10x volume of 10% buffered formalin		+	+		
Parasites alcohol (external), fresh water then alcohol/ formalin (internal)		+	+	+	
Stomach and **stomach contents** (for forage analysis and toxicological analysis		+	+	**?**	
Faeces (for parasitology and hormones)		+	+		
Skeletal samples*, teeth, baleen		+	+	+	+
Ear plugs, eyes		+	+		
Reproductive organs		+	+		
Heads (for auditory examination under MRI or CT scans) – hand removal required – not chainsaw; or in-situ ear extraction (femoral head disarticulator useful), fixed with formalin by the use of a syringe through the 'round window'. If not possible, immersion of whole ear bone in formalin is recommended.		+	+		

- for carcass codes (1-5) see below

? = marginal
NB by 48h carcasses will probably be at code 4, earlier in warm climates.

It is essential that samples are collected only from carcasses of sufficient quality. Some samples can be collected from decomposing carcasses, some only from fresh carcasses. Appropriate carcass codes are indicated throughout the protocols. **COLLECTION OF SAMPLES FROM INAPPROPRIATE CARCASSES IS A WASTE OF TIME AND CAN LEAD TO MISLEADING RESULTS.**

- **Code 1:** alive
- **Code 2 (carcass in good condition):** fresh/ 'edible'; fresh smell; minimal drying or wrinkling of skin or eyes; eyes clear; no bloating; tongue and penis not protruded; blubber firm and white; muscles firm, dark red and well defined, brain firm, little gas in intestines.
- **Code 3 (fair- decomposing but organs intact):** Some bloating may be present, possibly with tongue or penis protruded; mild odour, mucous membranes dry; eyes shrunken or missing; blubber blood tinged or oily, muscles soft and poorly defined; blood haemolysed; organs soft, friable and mottled but still intact; gut dilated by gas; brain soft and fragile with reddish cast but surface features distinct.
- **Code 4 (poor- advanced decomposition):** carcass collapsing; sloughing of skin; strong odour, blubber soft, possibly with pockets of gas or oil; muscle liquefying or easily torn, possibly falling off bones; blood thin and black; organs identifiable but very friable, easily torn and difficult to detect; gut gas-filled; brain soft, dark red, containing gas pockets, pudding consistency
- **Code 5 (mummified or skeletal remains):** skin may be draped over skeletal remains; any remaining tissues are desiccated

1.10 Processing of samples

1.10.1 Eye

Collect liquid and freeze (represents biochemistry at time of death)
Freeze lens (age)

1.10.2 Blood at time of euthanasia

Collect EDTA, Fl Ox, LiH and plain tubes (haematology, biochemistry, toxicology, hormones, and disease)

- Allow blood in plain tubes/ SST (serum tubes) to clot and for the clot to begin to shrink (room temp)
- Use EDTA blood to make 3 smears, chill remainder
- Centrifuge all remaining blood samples (not EDTA) at 3000 rpm for 10 minutes. If no centrifuge is available, allow samples to stand, chilled until the cells settle.
- Draw off clear liquid (plasma/ serum) with a pipette, keep 2 mL of Li Heparin plasma and 0.5 mL of Fl Ox plasma aside and divide remainder of all samples into 0.5-1 mL portions and freeze at -20 C or -70 C.
- Submit EDTA sample, the 2mL of Li Heparin Plasma and the 0.5 mL of Fl Ox plasma to lab as soon as possible (must be processed at lab within 48 hours, preferably within 24). Request full haematology and biochemistry profile.

1.10.3 Heart blood in plain tubes (disease, toxicology, hormones)

- Allow blood in plain tubes (serum tubes) to clot and for the clot to begin to shrink
- Centrifuge at 3000 rpm for 10 min. If no centrifuge is available, allow the blood clot to shrink at room temperature for a couple of hours. Draw off clear portion (serum), divide into 0.5- 1ml portions and freeze at - 20C or - 70C.

1.10.4 Chilled abnormal tissues (2-5cm cube in sterile container) (disease studies)

- If culturing facilities are close-by submit chilled ASAP
- Otherwise, sear surface of tissue by dipping a knife or spatula in alcohol, lighting it and, when it is hot, running it across the surface of the tissue.
- Slice into the middle of the tissue through the seared surface with a sterile scalpel
- Insert a sterile bacterial swab to swab the middle of the tissue, and then place the swab in culture medium
- Send to the lab, chilled, ASAP. If lab facilities are not accessible within 48- 72 hours, freeze the swab and tissue (the colder the better). Request bacterial culture (aerobic and anaerobic).
- Select a 5mm slice including healthy and diseased tissue and place in 10% formalin. Freeze remainder.

1.10.5 Swabs of affected sites in transport media or frozen **(disease studies)**

- Send bacteriological swabs in transport media to the lab, chilled, ASAP. If lab facilities are not accessible within 48- 72 hours, freeze the swab and tissue (the colder the better). Request bacterial culture (aerobic and anaerobic).
- Keep plain swabs frozen in case needed for microbial DNA.

1.10.6 Stomach and stomach contents (for forage analysis and toxicological analysis)

- One 70ml container of stomach contents and freeze, one 70ml container 3/4 stomach contents ¼ ethanol for biotoxin analysis (code 2 carcasses only)
- Freeze whole remainder GIT and contents.
- or: weigh full stomach, sieve stomach contents, dry otoliths and fish bones or spines, place beaks and fresh remains in 70% ethanol (Warneke 1997).
- Preserve parasites as below.

1.10.7 Chilled non- contaminated tissues (for virology and toxicology and fatty acid, stable isotope and hormone analysis)

1.5.7.1 Blubber:

- Trim off skin and edges of tissue with knife (prefer titanium-make note if stainless steel or any other knife used). Clean knife between tissues by wiping well with a new cloth and 100% ethanol.
- Three 1x 3 cm pieces of skin frozen for stable isotopes- freeze -20C
- Two 1cmx 1cm x 5mm deep pieces of skin, sliced up finely and placed in 10x vol saturated salt solution with 10%DMSO added or in 80% ethanol, or frozen if necessary.
- 1x1 cm by full thickness piece of blubber for fatty acids- freeze -20C
- 5x 100g full thickness pieces placed in glass or teflon or, if not available, wrap in aluminium foil then in plastic, and freeze (leave on the deep layers of the skin to assist with orientation of the sample later).
- 10g blubber for endocrinology- freeze

1.5.7.2 Muscle:

5mm cube sliced thinly and placed in 10x vol saturated salt solution with 10%DMSO added or in 80% ethanol, or frozen if necessary (code 2 only)

- 1cmx 1cm x 3 cm frozen (stable isotopes)

1.5.7.3 Other tissues :(liver, kidney, spleen, brain)

Trim off edges of tissue with titanium knife (titanium preferred- make note if stainless steel or any other knife used). Clean knife between tissues by wiping well with a new cloth and 100% ethanol.

- Freeze 100g in glass/ Teflon (organics- not kidney or spleen), 100g in plastic (biotoxins-code 2 only- not spleen), 20g in plastic or glass (inorganics- not spleen) and 1cm x 1cm x 3cm in plastic (stable isotopes).
- Freeze remainder for virology/ bacteriology –70C if possible.

1.10.8 Parasites

Nematodes 10% formal saline or 70% ethanol, Cestodes and trematodes- relax in fresh water first then in formal saline

1.10.9 Faeces (for parasitology and hormones)

Freeze some and place some in 10x vol of 10% formalin. If animal code 2, freeze a small amount for DNA forage analysis.

1.10.10 Skeletal samples, teeth, ear plugs (taxonomy, life history, ear trauma)

- If entire skull- consider removal of ears (Kettern protocol)
- Place ear plugs in 10% formalin
- Teeth, baleen, skeletal samples, flippers – freeze

In the case of a mass stranding, if the jaws and teeth are to be dealt with immediately, check that all bags are properly sealed – if they are not there is a chance teeth will be separated from the original jaw. (Otherwise keep them cool until they can be frozen)

1.10.11 Reproductive organs

- collect **gonads**- weigh and measure both (Testis- weigh and measure testis and epididymis separately (one only); Examine ovaries for CL/ follicles, weigh and measure and keep chilled

Rt Gonad: Weight_______, _______x_______x_______, Features_____________
L Gonad: Weight_______, _______x_______x_______, Features______________

- Examine **tract** for signs of current or previous foetus. Measure uterine wall thickness, cervix, int diam and length uterine horns. Keep chilled. (Rowles 2001)

Uterus: wall__________, int diam__________, length___________
Cervix____
(for mass strandings use data form)

- If testis or uterus are very large, take a cross- and longitudinal section of the testis (Rowles 2001) and representative portions of the uterus (vagina, cervix, uterine horn, fallopian tube)

Code 2 carcass- select 1 cm wide samples of vagina, uterus and fallopian tube or testis and epididymis and place with other tissues in 10% formalin for histoanatomy.
Cut vas deferens, testis and epididymis and make smears (Warneke 1997).

1.10.12 Measurements and morphological description

Check data is complete and back up.

1.10.13 Photos/ description

Check all data is complete and back up.

1.10.14 Gross pathology photos/ description

Check organ descriptions/ photos against photo library of normal specimens and add amendments if necessary.
Check all data complete and back up.

1.10.15 All organs and edges of lesions < 1cm thick slices in 10x volume of 10% buffered formalin (for histo-anatomy/ histo-pathology) (disease studies)

Arrange to have tissues transported to a pathology lab to be trimmed and embedded ASAP (Long periods of fixation do not affect routine histopathology but shorter periods of fixation (2-7days) are often better for immunohistochemical and in- situ hybridization studies, which are becoming more widely used).

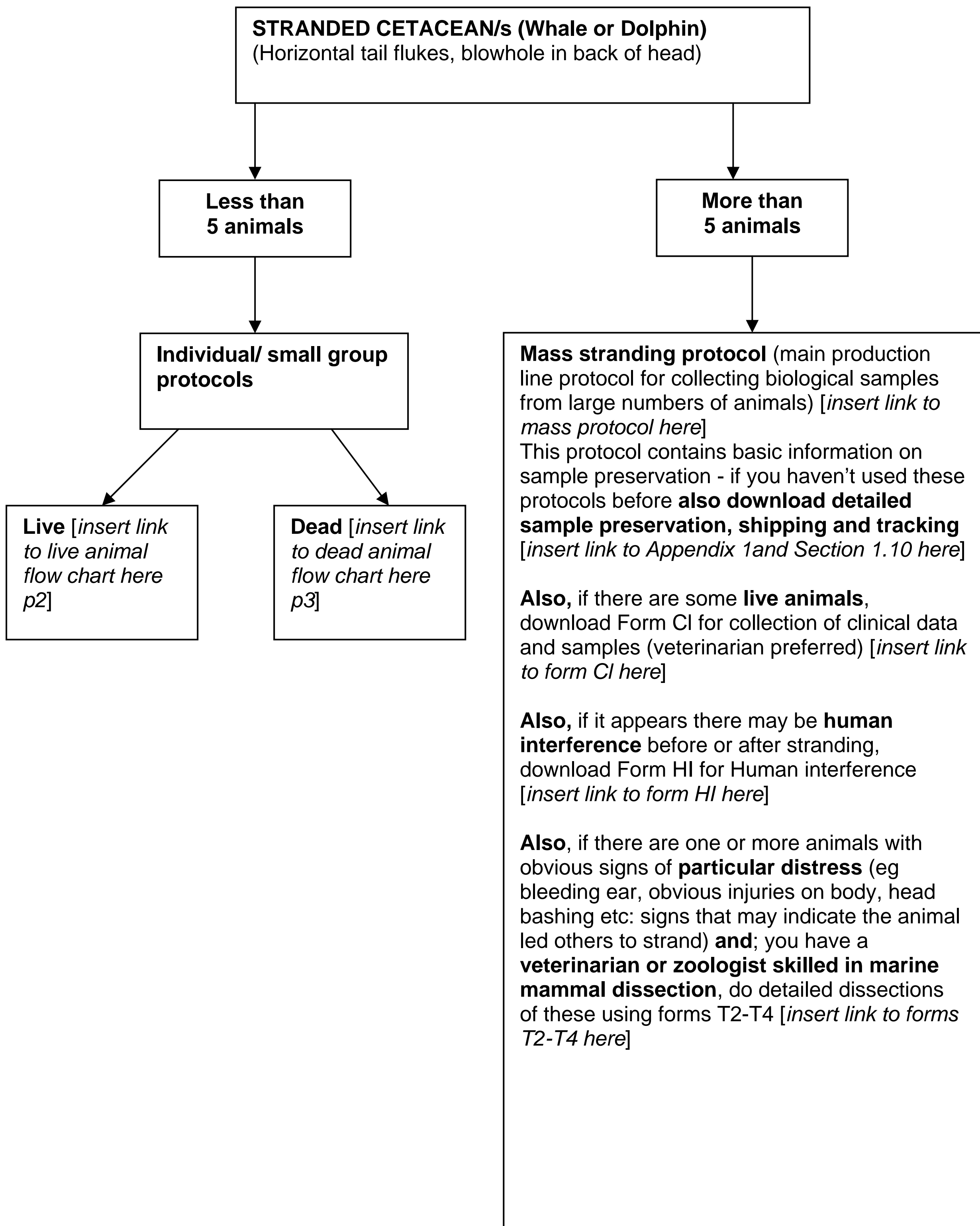
1.10.16 Label all samples

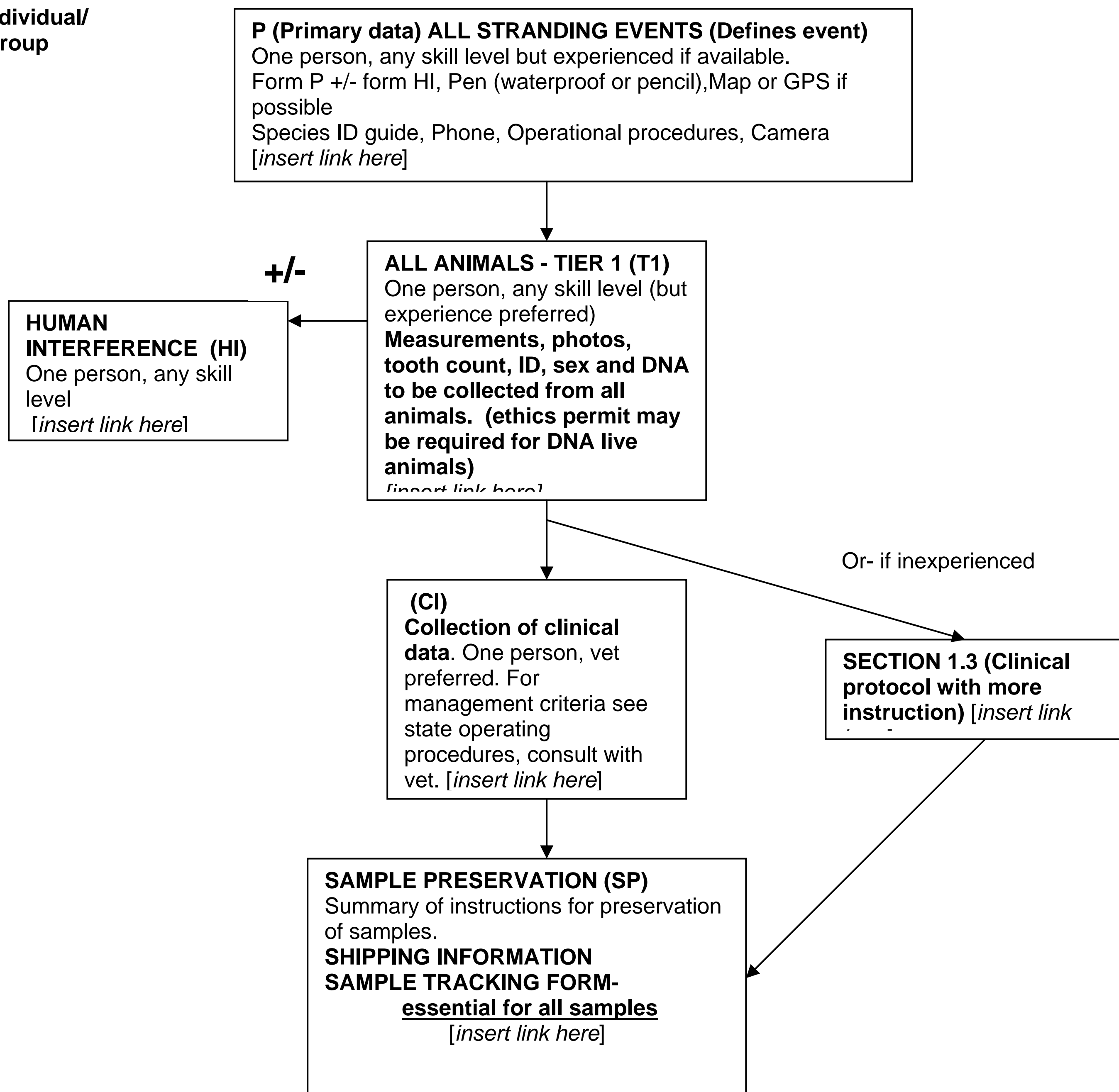
Have a label with the whale’s number, the sex, the date and the location of the stranding written on it. Check all samples against the records in the kit notebooks.

2 HOW TO CHOOSE YOUR LEVEL OF SAMPLING

FOLLOW LINKS IN FLOW CHART. CHECK EQUIPMENT LISTS AND SAFETY RECOMMENDATIONS IN THE PROTOCOLS

THESE PROTOCOLS RELATE TO SAMPLING ONLY. FOR ALL OTHER ASPECTS OF STRANDING MANAGEMENT REFER TO OPERATING PROCEDURES FOR YOUR STATE. NB PERMITS FROM YOUR STATE CONSERVATION AGENCY (AND ANIMAL ETHICS PERMITS IF LIVE ANIMAL) ARE REQUIRED FOR SAMPLES TO BE COLLECTED.





Form P- one form per event. Remainder- one form per individual (except mass stranding protocol).
WHEN IN DOUBT CALL THE RELEVANT STATE OR COMMONWEALTH GOVERNMENT AGENCY.
SEE ALSO: State operating procedures, register of facilities and researchers (Appendix 2), guide to dependent young (Appendix 3) and species ID key (Appendix 4).

DEAD individual/ small group

P (Primary data) ALL STRANDING EVENTS (Defines event)
One person, any skill level but experienced if available.
Form P +/- form HI, Pen (waterproof or pencil),Map or GPS if possible
Species ID guide, Phone, Operational procedures, Camera
[*insert link here*]

ALL ANIMALS - TIER 1 (T1)
One person, any skill level (but experience preferred)
Measurements, photos, tooth count, ID, sex and DNA to be collected from all animals. (ethics permit may be required for DNA live animals)
[*insert link here*]

+/-

HUMAN INTERFERENCE (HI)
One person, any skill level
[*insert link here*]

TIER 2 (T2) external sampling
2 people, some skill preferred.
Blubber thickness, skin, blubber, muscle, head, mammary
(life history, DNA, toxins, stable isotopes, fatty acids, ear/ skull studies, ext. parasites).
[*insert link here*]

(**PREFERRED ALTERNATIVE**)
Transport for thorough post-mortem by vet, pathologist or zoologist trained in cetacean dissection (including zoonoses). Ideally pathologist and cetacean zoologist.

TIER 3 (T3) basic internal sampling
At least 2 people, one with experience in cetacean dissection.
Kidney, liver, spleen, blood for toxicology/ serology, reproductive organs, digesta, internal parasites
[*insert link here*]

Single animal post-mortem protocol (**SECTION 1.4**). Comparable to T2-T4 but with more detailed dissection. (May choose to record on forms T1-T4 or within detailed protocol). For use as an alternative where there are unlimited time and resources, of for additional instruction for those using T2-T4 form based protocols.

TIER 4 (T4) detailed internal sampling
At least two people, one vet or zoologist experienced in cetacean dissection (including understanding of zoonoses). Complete sampling and examination of internals for research and pathology.

SAMPLE PRESERVATION (SP)
Summary of instructions for preservation of samples.
SHIPPING INFORMATION
SAMPLE TRACKING FORM-
<u>essential for all samples</u>
[*insert link here*]

Complete information on sample preservation and storage **(SECTION 1.10 and Appendix**

Form P- one form per event. Remainder- one form per individual (except mass stranding protocol).
SEE ALSO: State operating procedures, species ID key, register of facilities and researchers, specialist protocols, guide to dependent young, introduction to protocols, equipment list.

HOW TO CHOOSE YOUR LEVEL OF SAMPLING

SEE FLOW DIAGRAM AND EQUIPMENT LISTS

THESE PROTOCOLS RELATE TO SAMPLING ONLY. FOR ALL OTHER ASPECTS OF STRANDING MANAGEMENT REFER TO OPERATING PROCEDURES FOR YOUR STATE.

1. **Primary stranding data (form P) and basic measurements, photos, check for human interference, and DNA samples (form T1) to be collected, on the beach, from all animals under all conditions.**
2. Form P describes the event (one form per event), the remainder describe individuals- therefore one form per individual (except mass stranding protocol, Section 5)

When P completed

1. If you have **less than 5 animals,** and sufficient **personnel** and **equipment** (below) proceed to **forms T1-4** as appropriate (Additional forms where appropriate for human interference or live animals (HI and Cl) See Appendix 1)
2. Generally- complete P and T1 for all animals, then T2 for all animals, then T3, etc. rather than completing all on one animal before moving to the next animal.
3. If **more than 5 animals**, and 6 people (some experienced) consider using mass stranding protocol (Section 5), which is set up as a "production line" (T1 information is contained within this protocol)
3. If you have a **single animal which can be transported** for post mortem, do so, and use T1-T4 or the detailed protocol (Section 4).

THROUGHOUT- PAY ATTENTION TO CARCASS CODES (form T1) AND CORRECT METHOD OF PRESERVATION. Don't waste time collecting samples from animals unless they are of good enough carcass quality and you have the means to preserve them properly. PAY ATTENTION TO LABELLING- ensure it is permanent- label inside and out, preferably double bag and put a label between the bags.
MAKE SURE YOU INCLUDE A LABEL AND SCALE IN ALL PHOTOS.

REQUIRED EQUIPMENT
SAFETY
General safety and public health for stranding events are covered in the operating procedures of each state. Safety precautions pertaining to collection of samples generally consist of basic hygiene and safety around sharp objects:

- Lots of drinking water
- Broad brimmed hat and sunscreen/ wind and rain protection
- Good hygiene
- Double glove and apply barrier cream to skin
- Overalls
- Face mask and goggles if near blowhole or if tuberculosis is suspected
- Boot chains/ instep crampons for larger animals (to avoid slip accidents)
- Steel cap boots
- Cover sharp bone fragments
- Chain mail gloves (optional- only if fit correctly)
- Knife sharpeners (stones as well as steels) and alcohol rags to clean knives

- Sharps container and/ or knife block
- Disinfect all cuts with iodine or alcohol 70%
- Disinfect all equipment after use with broad-spectrum disinfectant.
- Safety data sheets should accompany all chemicals eg fixatives, preservatives.

PRIMARY STRANDING DATA (P) and HUMAN INTERFERENCE (HI)
Form P +/- form HI, Pen (waterproof or pencil), clipboard
Map or GPS if possible, camera (prefer digital) and label with scale attached (for inclusion in photo)
Species ID guide, Phone, Operational procedures

CLINICAL DATA (Cl) -data collection- not a guide to triage/ management
Form Cl, Pen (waterproof or pencil), Clipboard, Watch, PERMANENT labelling pen
Optional- thermometer (extended probe, non glass), Blood collection tubes, needles, syringes, eskies, ice/ refrigeration, ethanol 80% or saturated salt solution, glass or unbreakable non- leak plastic vial.

TIER 1 SAMPLING (T1)-Measurements, photos, tooth count, ID, sex and DNA to be collected from all animals).

Form T1, Pen (waterproof or pencil), Camera (prefer digital), Tape measure, accurate scales. Plastic scourer/ scalpel blade, Glass or unbreakable plastic vial, 80% ethanol or saturated salt solution +/-DMSO, or freezer. PERMANENT labelling pen (recommend Parcelmate black)

TIER 2 SAMPLING (T2) - Blubber thickness, skin, blubber, muscle, head, mammary

Equipment for Tier 1 plus:
Form T2
Large/ flensing knives, sharpening stone and steel, Meat hooks
Ruler/ callipers, Plastic bags (large and small), stack canisters/ plastic containers, Ice, eskies, Labelling gear (Dymo tape or pencil and card for internal labels (between double bags)). PERMANENT labelling pen- recommend Parcelmate black.
Gloves, overalls

Off beach (preservation)

Plastic bags (zip lock), Teflon bags/ sheeting (preferred) or alfoil
Stainless steel blades (titanium preferred), 100% ethanol, cutting board. Labelling gear Dymo tape or pencil and card for internal labels (between double bags). PERMANENT labelling pen- recommend Parcelmate black.
Freezer.
Forms SP, sample tracking form, Appendix 4.

TIER 3 SAMPLING (T3) - Kidney, liver, spleen, blood for toxicology/ serology, reproductive organs, digesta, internal parasites

Equipment for Tier 1, 2 plus:
Form T3
Dissection instruments
10% buffered formalin and well- sealed sample jars/ buckets
100% ethanol (70-80% if not available)

Sieve for stomach contents if too large to store whole.
Consider facemasks, eye protection, chain gloves, crampons/ boot chains.

TIER 4 SAMPLING (T4) - complete examination and sampling for pathology and research

Equipment for Tier 1, 2, 3 plus:
form T4
Bacto swabs, forceps, scissors, scalpels, sterile sample containers, microscope slides, blood tubes
String to tie off gut, Facemasks, eye protection

3 LIVE ANIMAL CLINICAL EXAM AND SAMPLING PROTOCOL

3.1 Initial assessment

Any assessment must be rapid yet thorough enough to enable classification according to the following:

- **Dead** - post mortem or disposal
- **Rescue attempt**
- **Requires treatment** prior to rescue attempt
- **Possible euthanasia**
- **Moribund or impossible/impractical to rescue** - candidate for euthanasia

Whales should be identified by an accepted, failsafe system, known to all. Use of cattle tags on cord tied around tail stock has proven successful in Tasmania. Spaghetti tags may be useful for monitoring re-float attempts. Colour coding may be useful at live strandings, for example:

- **White** - dead
- **Green** - apparently healthy
- **Yellow** - minor incapacity
- **Red** - major problems/euthanasia

3.2 Clinical examination

NB: Be wary of too frequent monitoring of clinical signs: measure simple observations such as breathing rate and character constantly, and the other things either as the opportunity arises (eg because you need to do some other procedure) or if there is a change in observed signs, which means that there may be changes in other signs as well.

3.2.1 General condition

external lesions, dorsal and cervical muscle/blubber mass.

3.2.2 Reflexes

Presence or absence:

- Blowhole – should be closed or should close if touched;
- Palpebral – should have reflex lid closure to touch. Also note any discharges;
- Anal – tightening should be elicited by touching the surrounding area.

3.2.3 Respiratory Rate & Quality

- Rate of one to three per minute;
- There may be long periods of apnoea especially if the animal is depressed or comatose;
- If the rate rises above 10 when stressed, eg by handling (or alternatively dropped below it once made comfortable and stress reduced) but doesn't stay high, it is a good sign. A consistently high rate carries a very bad prognosis. (pers comm. Ian Robinson IFAW).

3.2.4 *Heart Rate*

- Auscultate or palpate, posterior to pectoral flipper a the level of the carpus (generally seen as a bend in the caudal border of the flipper).
- Heart rate may be very slow or weak and difficult to determine.
- Failure to exhibit tachycardia on inspiration is a grave prognostic sign.

3.2.5 *Muscle Tone*

assessed by :

- Attempting to open the mouth or by resistance to traction of the tongue;
- The ability of the whale to move.

3.2.6 *Skin*

- The condition will vary depending on the time of exposure.
- Note - drying, cracking, blistering, sloughing.
- Other traumatic injuries.
- Response to touch - may be quite violent with sunburn injury.

3.2.7 *Body Temperature*

- Only useful if suitable rectal probes are available (minimum 30 cm in length). Do not use glass.
- Normal values 36.5 – 37.5 C (97.7 – 99.5 F).

3.2.8 *Vocalisations*

- Audible sounds from blowholes apart from respirations.
- Whistles and high-frequency squeals while possibly associated with stress may be useful clinical indicators. Vocalisations diminish or cease in depressed animals.

3.2.9 *Other Considerations*

- Respiratory distress from water or sand inhalation or airway obstruction.
- Bleeding from any orifice or wound.
- Response to cooling.
- Evidence of being buried in sand.
- Response to return to the sea.

3.3 Collection of data / samples

Measurements and morphological description: use this form (CI) in conjunction with general stranding and morphometric data forms (Forms P and T1)

Photographs: use colour slide film (or digital if possible) and avoid using flash. Put the animal's ID and date and scale in the picture but not over any detail. Take all photos at right angles to the subject wherever possible and use a backboard where possible. (Kemper, unpub.) (Use forms P and T1)

Skin: “2x cheese grater scrapes”1.5-3mm deep in 10x vol of saturated salt solution +/- 10% DMSO or in 10x vol ethanol 80%

Biopsies of lesions: 5mm thick slices/ punch biopsy in 10% buffered formalin or by fine needle aspirate.

Blood in EDTA, Fl Ox, LiH and plain tubes - preferably at least twice during event (see diagrams from Geraci and Lounsbury, 1993)

Faeces: some in 10% buffered formalin, some frozen

Swabs/ blowhole spray: in the case of single stranded animals or where the individual is thought to be diseased, microbiology or cytology samples can be collected by swabbing (swabs of affected sites placed in transport media or smeared on glass slide, respectively) or placing a sterile agar-coated petri dish or microscope slide, respectively, over a wiped blowhole (Sweeney and Reddy, 2001).

Use form below in conjunction with stranding and morphometrics forms P and T1

4 SINGLE ANIMAL ON-BEACH POST-MORTEM PROTOCOL

4.1 Introduction

This protocol is to be applied for single animal strandings.

As a minimum, forms P and T1 are required.

Notes:

i. If you do not have facilities to store or preserve the samples properly **do not collect them.**

ii. **If any step cannot be completed, mark "not done" on the form and explain why.** This feedback helps to identify areas where training, equipment or modifications to the protocol are needed.

iii. After completion of the protocol, process samples as described in Section 1.10.

4.2 External examination

4.3 Photographs – see T1 form (below for detail)

4.4 Body measurements based on SA Museum protocol (Kemper, unpublished) – see T1 form.

4.5 Photographs (for all stages of carcass condition)

Use digital camera or colour slide film if possible and avoid using flash. Put the animal's ID and date and scale in the picture but not over any detail. Take all photos at right angles to the subject wherever possible and use a backboard where possible.

1. **Whole animal side view** L#_________ R#__________
2. **Tail flukes** #__________
3. **Dorsal fin** #__________
4. **Head from side** L#_________ R#__________
5. **Head from above** #__________
6. **Teeth/ baleen** #__________
7. **Whole ventral surface** #__________
8. **Genital slit, anus and umbilicus (all in one)** #__________
9. **Scars, wounds, injuries, other abnormalities** #__________
10. **Flipper** L#_________ R#__________
11. **External parasites(check grooves and orifices)** #__________
12. **Any other lesions during dissection** #__________

4.6 Tooth counts (for all stages of carcass condition)

- **<u>Count teeth</u>** from front to back and enter any missing teeth in sequence i.e. 10 (2) 9 (4) is 10 teeth followed by 2 missing, followed by 9 present, etc.
- Note if teeth not erupted.

UR ______________________ UL _____________________

LR ______________________ LL _____________________

____

____

____

4.7 External parasites (for stages of carcass condition 1-4)

- **<u>Check all orifices and grooves for parasites</u>**
- **Collect** in 10% formalin or 70% ethanol
- Label with animals ID and the location that the parasite was found on the body.

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4.8 External assessment (for all stages of carcass condition)

- Is the skin intact or peeling?
- Note any scars, wounds, lesions or other irregularities, mark them on sketches and photograph them with a cross-referenced label in the photograph.
- Note any bloating of the carcass or discharges from orifices, sunken eyes, prominent vertebral processes or sunken appearance to the neck and dry or haemorrhagic mucous membranes.
- **<u>Assess the condition of the carcass</u>** based on the 1-5 system of Geraci and Lounsbury (1993)

Code 1: alive

Code 2 (carcass in good condition): fresh/ edible; fresh smell; minimal drying or wrinkling of skin or eyes; eyes clear; no bloating; tongue and penis not protruded; blubber firm and white; muscles firm, dark red and well defined, brain firm, little gas in intestines.

Code 3 (fair- decomposing but organs intact): Some bloating may be present, possibly with tongue or penis protruded; mild odour, mucous membranes dry; eyes shrunken or missing; blubber blood tinged or oily, muscles soft and poorly defined; blood haemolysed; organs soft, friable and mottled but still intact; gut dilated by gas; brain soft and fragile with reddish cast but surface features distinct.

Code 4 (poor- advanced decomposition): carcass collapsing; sloughing of skin; strong odour, blubber soft, possibly with pockets of gas or oil; muscle liquefying or easily torn, possibly falling off bones; blood thin and black; organs identifiable but very friable, easily torn and difficult to detect; gut gas-filled; brain soft, dark red, containing gas pockets, pudding consistency

Code 5 (mummified or skeletal remains): skin may be draped over skeletal remains; any remaining tissues are desiccated

4.9 Measurements (codes 1-4)

Complete **external measurements** NB do not measure 1/2 girth or tail width and double it; if you have to do this, record the 1/2 measurement and note it as such.

1.TOTAL LENGTH (tip of upper jaw to deepest part of fluke notch)		
2.Tip upper jaw- centre of eye		
3.Length of gape (upper jaw to corner mouth)		
4.Tip upper jaw to blowhole		
5.Tip upper jaw to front insertion of flipper		
6.Tip upper jaw to tip dorsal fin		
7. Tip upper jaw to centre anus		
8.Max girth		
9.Flipper- tip to front insertion		
10.Flipper- max width		

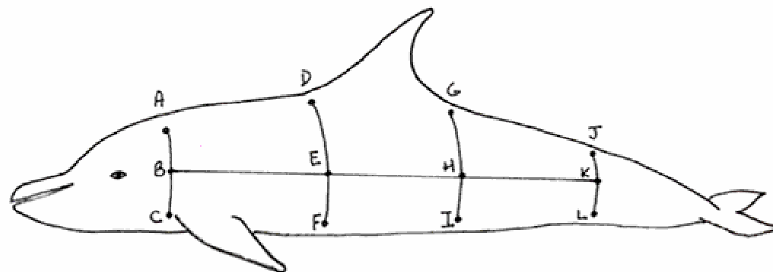
11.Tail flukes tip to tip		
12. Depth of fluke notch		
13.Dorsal fin tip to base		
Throat grooves? _____ feathering of tongue? _______Snout hairs?______	(Y/ N)	
Weight		

4.10 Initial sampling (Forms T2-T4 may be required)

If liquid N available and animal *less than 2 hours dead*, sample skin and liver (if possible in time) and freeze immediately in liquid N (for mixed function oxidase analysis- toxin exposure).

Incise through the blubber to the muscle according to the diagram below and measure **blubber thickness** at the specified locations. For compatibility with SA museum, ensure thicknesses mid-dorsal just in front of dorsal fin and mid-ventral just in front of umbilicus are measured.

Blubber measurements (codes 2-4): refer to diagram. Measurements to be taken with vernier callipers and stated in millimetres to the nearest 0.1mm. Measure from the base of the skin to the surface of the muscle.



A	B	C	D	E	F	G	H	I	J	K	L

(From K. Evans: protocol for single stranded cetaceans)

4.11 Collection of samples

1. **Swab any orifices with discharges** (code 2) and as deep as possible inside the blowhole using a sterile swab and place it in its accompanying transport medium.
2. Excise any **external lesions** (codes 2-3) and hold chilled in a sterile container.
3. If you see any **parasites** collect as many as possible into a jar of fresh water noting where they were found. If they are embedded, cut the surrounding tissue out. Put any associated lesions in 10% formalin.
4. Excise a 20cm x 20cm x full thickness block of **skin and full thickness blubber** (codes 2-3) and hold chilled. (location to be decided- currently anterior to dorsal fin)
5. Excise a 6cm x 6cm x 6 cm **block of muscle** (codes 2-3) from under site of blubber sample, and hold chilled
6. Remove an **eye** (codes 2-3) and hold chilled
7. Remove **head** (codes 2-3) intact, **Don't use chainsaw.** Examine oral cavity for abnormalities and hold chilled. Alternatively, remove **teeth/ jaw piece** (mid left mandible 4-5 teeth with roots intact – codes 2-5) **or baleen** (one side or several of longest complete – codes 2-5) and **ear** (codes 2-5) as per Kettern (to obtain protocol and training). NB: cutters work better on bone if you cut through soft tissues first using a knife.
8. Remove a **flipper** (codes 2-5)

Swabs ____

Lesions ____

Ext parasites ____

Skin with blubber __________ **Muscle** __________ **Eye** ________ **Head** ________

Skin _______ Jaw _______ Baleen ________ Teeth __________ Flipper ________

4.12 Internal examination

Samples only to be collected from code 2-3 carcasses, except for stomach contents and reproductive tracts (2-4).

Record internal samples on Forms T3 & T4.

4.13 Dissection (based on Duignan, 2000)

1. Flense the carcase down to the muscle by removing the blubber in convenient sized blocks using large knives, flensing knives and hooks. Initial skin incisions may be best made with a scalpel to avoid blunting knives.

2. Examine the blubber for **parasitic cysts**, estimate the number of cysts and collect some parasites and place in fresh water (*Phyllobothrium* sp., a cestode or tapeworm).

   Parasites ____

   Sampled ________

3. Check **muscles, subcutis** for lesions (oedema (fluid)/ haemorrhage). NB the muscles are commonly very dark in colour so bruising may be difficult to detect. Look for irregular patches of colour change, or bloody, gelatinous or gaseous patches.

   Muscle/subcut ____

4. Locate the **mammary glands** cranio-lateral to the genital slit. Open from the nipple and look for any **parasites** in the ducts. Note, photograph and put aside in a sterile container any **irregularities** (they should be uniformly pale pink).

   Mammary/ parasites ____

   Sampled ________

5. Cut off the **flipper** and **scapula** (these have no bony attachment with the rest of the thorax)
6. Find the **axillary lymph node**, section it and put it aside for pathology sampling (in a sterile container if it appears reddened or cheesy). It should be creamy grey-beige and have even consistency. (Lymph nodes indicate if an infection has been present and often infectious agents lodge here)

   Axillary LN ____

   Sampled _________

7. If any of the **brachial nerves** are visible, collect some and put aside with the lymph node.

   Brachial N ____

   Sampled _________

8. Separate the **ribs** at their attachment to the spine and sternum, taking care not to damage them if the entire skeleton is to be kept (see 5.9 Skeleton prep.), thus **opening the thorax**. If there is excessive, coloured or cloudy fluid, strands or tags of fibrin or adhesions, collect **fluid** in a sterile syringe or tissue in a sterile container or **swab** the cavity with a sterile swab and place it in its accompanying medium. Hold chilled.

   Thoracic cavity ____

   ____

   ____

____

____

Sample__________

9. Remove the **abdominal wall** taking care not to puncture the gastro-intestinal tract. If there is excessive, coloured or cloudy fluid, strands or tags of fibrin or adhesions, collect **fluid** in a sterile syringe or tissue in a sterile container or **swab** the cavity with a sterile swab and place it in its accompanying medium. Hold chilled.

   Abdominal cavity ____

   ____

   ____

   ____

   ____

   Sample__________

4.14 Observation, bacteriology and photos

1. **Stand back and observe** the layout, colour, texture, size and regularity of the organs and body cavities. Note and photograph anything you think looks unusual. (compare with photos or diagrams of "normal" animals if available)
2. **Photograph** the abdominal and thoracic cavities
3. If possible push the gastro-intestinal tract to the side to get glimpse of any deeper organs, such as the kidneys and reproductive tract.
4. Leaving some of the edge of the lesion behind for formalin fixation, excise a (5 x 5 x 5 cm) piece of any **potential lesions** (if the lesion is smaller, take surrounding tissue as well), including the edge of the lesion if possible, and place in a sterile container.

   Comments ____

   ____

   ____

   ____

   ____

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   ____

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Samples (lesions in sterile container) ________________________________

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____

4.15 Thorax

(Adapted from Duignan, 2000)

If **any abnormalities** are seen during the following steps, immediately collect **fluid** in a sterile syringe or tissue in a sterile container or **swab** the fluid with a sterile swab and place it in its accompanying medium or, for **tissues**, leaving some of the edge of the lesion behind for formalin fixation, excise a (5 x 5 x 5 cm) piece (if the lesion is smaller, take surrounding tissue as well), including the edge of the lesion if possible, and place in a sterile container. Hold chilled.

Lesions collected____

____

____

1. Tie off the **oesophagus** at its entry to the stomach.
2. Examine the sac around the heart (**pericardium**). If there is excessive, coloured or cloudy fluid, strands or tags of fibrin or adhesions, collect **fluid** in a sterile syringe and keep chilled.
3. **Aspirate blood from the heart** with a sterile syringe and place in a plain or serum separator tube. If it hasn't clotted put a few ml in an EDTA tube. (Heart blood can be used to detect exposure to various infectious agents and some toxins)

   Heart Blood_________

4. Locate the **thyroid**. It is a bi-lobed organ, at the base of the trachea, ventrally. Put it aside. (The thyroid regulated the animal's metabolism.)
5. If it is a young animal, locate the **thymus** (a shapeless organ between the heart, sternum and thyroid) and put it aside. (The thymus is where much of the animals immune system develops)
6. If possible, **remove the whole respiratory tract, heart and oesophagus**, starting by dissecting out the tongue, cutting through the hyoid bones that support the larynx and working down into the chest cavity.
7. Open the **oesophagus** and examine.

   Oesophagus____

   ____

8. Open the **trachea** and follow the **airways** down as far as possible then make many cuts through the flesh of the **lung**, looking for any **irregularities**.

   Respiratory tract____

   ____

   ____

   ____

9. In examining the **heart** look for **tags, growths, roughening** or **discolorations** on the internal and external surfaces and valves and **parasites** within the chambers.
10. Using scissors or a knife, follow the flow of blood from the vena cava to the right atrium, through the right AV valve to the right ventricle and then up through the pulmonic valve to the pulmonary artery.
11. Then follow the pulmonary vein into the left atrium, cut through the left AV valve to the left ventricle and then up through the aortic valve into the thick muscular aorta.
12. Cut the heart into slices, looking for any changes in colour of the heart muscle.

    Heart____

    ____

    ____

    ____

13. **Put all organs aside.**

4.16 Gastrointestinal tract

1. Tie off the **oesophagus** where it enters the stomach if not already done, and cut ABOVE the tie. Tie the **small intestine** twice where it leaves the stomach and cut between the ties.
2. Remove the <u>**stomach**</u> by cutting through its attachments and bag it (be sure to put the **spleen and pancreas aside**). If the stomach is too big to remove then collect sample of stomach contents.
3. Cut through the attachments of the intestine to the dorsal abdominal wall and remove the **intestines** and bag them. At this stage note the **mesenteric lymph nodes** where the intestines attach to the body wall. Remove some and put them aside. If the GI tract is too big to remove fully then remove representative samples of each section – duodenum, jejunum, etc.

   GI Tract ____

   ____

   ____

   Samples______________________________________

4.17 Abdomen – observations, bacteriology and photos

1. Stand back and observe the layout, colour, texture, size and regularity of the organs and abdominal cavity. (Compare with photos or diagrams of "normal" animals if available.) **Note** and **photograph** anything you think looks unusual.
2. Photograph the **abdominal cavity**.
3. Leaving some of the edge of the lesion behind for formalin fixation, excise a (5 x 5 x 5 cm) piece of any **potential lesions** (if the lesion is smaller, take surrounding tissue as well), including the edge of the lesion if possible, and place in a sterile container.
4. If not sampling for pathology – slice the **liver** and open the **bile ducts** and examine for **irregularities** and **parasites**. Lift the **kidney** from the abdominal wall and follow the **ureters** to the **bladder**. Slice the kidney and examine for irregularities and parasites.

   Abdominal cavity and organs ______________________________________

   ____

   ____

   ____

   ____

   ____

   ____

   ____

   Samples of lesions in sterile containers______________________________

   ____

   ____

4.18 Organ samples for trimming and freezing for toxin and forage studies (isotopes, fatty acids), genetics, virology (codes 2-3)

Do not remove the organs yet.

1. Collect **500g pieces of liver (left caudal), kidney (left caudal), spleen, lung, brain.** NB: if the skull is important (see list) take "core sample" via the foramen magnum and put some aside for formalin fixation as well. If possible it may be best to remove the whole head from the beach and remove the brain at another location where there are the tools, time and expertise, to minimise damage to the skull and ear structures. Place samples in plastic bags (Teflon preferred).
2. Aspirate **urine** in syringe and label, chill.
3. Aspirate **bile** and chill away from light.

Samples: Liver _______ Kidney _______ Spleen _______ Lung _______

Brain _______ Urine _______ Bile ________ Head _________

4.19 Pathology samples

Should be no thicker than 1 cm. Place in a clean jar for fixation in 10% buffered formalin (1 vol of tissues to 9 vol of formalin – can be added now, or tissues kept chilled until formalin is added in the lab a few hours later). When sampling lesions the priority is for tissue at the junction of the diseased and normal tissue as this is usually where the most diagnostic action is taking place. It is important to sample a full range of tissues as even tissues of normal appearance may contain microscopic changes that may either be diagnostic themselves or assist in the interpretation of visible lesions. Sample:

1. All **potential lesions** found so far.

   Samples____

   ____

2. The **tissues you put aside**:

   - Axillary lymph node and brachial nerve
   - Thymus and thyroid
   - Spleen and pancreas
   - Lung- some from the edge and some from the centre, include some airway
   - Pleural lymph nodes- on the caudal border of the lung. Slice lengthwise.
   - Heart- slices of left and right ventricle walls, septum and any abnormalities
   - Mesenteric Lymph node- slice lengthwise
   - Brain if possible (whole brains are best fixed whole and then sliced when fixed).

   Samples: Axillary LN _______ Brachial N _______ Thymus ________

   Thyroid _______ Spleen _______ Pancreas ______ Mesenteric LN ______

   Lung _______ Pleural LN _______ Heart _______ Brain ________

3. And from the carcass:

   - **Adrenals –** 2-15cm long, craniomedial to the kidneys under the (sometimes thick) peritoneal membrane. Slice them lengthwise. (Adrenals can indicate if the animal has been exposed to a stressful event prior to stranding.)
   - **Liver –** make many cuts through the substance of the liver and examine. Sample. Check in the ducts for parasites.
   - **Kidneys –** pull off abdominal wall and keep pulling to follow the ureters to the bladder. Make several cuts in the kidneys and examine. Sample.
   - **Bladder** wall

- Examine the **reproductive tract** and sample any **irregularities**. Otherwise leave it for later.

Samples: Adrenals _____ Liver _____ Kidneys ______ Bladder _______

Reproductive tract lesions _______

4.20 Reproductive tract

1. Remove **entire reproductive tract** for processing later OR if too large collect **<u>gonads</u>** – weigh and measure both: weigh and measure testis and epididymis separately (one only); examine ovaries for CL/ follicles, weigh and measure and keep chilled

   Rt Gonad: Weight________________, _______x_______x_______,

   Features____

   L Gonad: Weight________________, _______x_______x_______,

   Features____

2. Examine **tract** for signs of current or previous foetus. Measure uterine wall thickness, cervix, int diam and length uterine horns. Keep chilled. (Rowles, 2001)

   Uterus: wall __________ int. diam. __________ length ___________

   Cervix ____

3. If testis or uterus are very large, take a cross- and longitudinal section of the testis (Rowles 2001) and representative portions of the uterus (vagina, cervix, uterine horn, fallopian tube)

   Samples____

4.21 Skeleton preparation

As a guide, the South Australian Museum requests the following (Kemper, unpub.):

Contact your local museum or coordinator for guidance on priority species and method of preparation.

Whole skeleton/ body – very important:

- All bottlenose and common dolphins
- Southern right whale (especially adult)
- Pygmy right whale
- False killer whale
- Risso's dolphin
- Killer whale

Whole skeleton – if possible

- Fin whale
- Adult humpback whale
- Beaked whales
- Dwarf sperm whale

Less Important - try to collect **head**

- Other species
- Gulf dolphins (Sth Australia)

4.22 Sample preservation

Preserve samples as outlined in Section 1.4: Processing of Samples.

5 MASS STRANDING ON-BEACH POST-MORTEM AND SAMPLING PROTOCOL

This protocol is modified from that written by Karen Evans, an adaptation of Thiele, (1996).

5.1 INTRODUCTION

- This is a "production line" protocol for use with 5 or more animals and 6 or more people (some with experience) recommended for mass strandings.
- It should be used with Forms P, T2-T4.
- As a rough guide, we suggest the following priority for samples. If your state authorities have alternative priorities, follow those.
- Read Section 1.10, and refer to Appendix 1 –**SUMMARY PRESERVATION, SHIPPING and TRACKING Form**. **If you do not have facilities to store or preserve the samples properly do not collect them.**
- **Within the protocol, numbers in brackets (eg (2-3)) indicate suitable carcass code for those samples.** Also refer to carcase condition guide (in overview).
- After completion of the protocol, **process samples as described in Section 1.10**

ORGANISER Form P (Primary data) ALL STRANDING EVENTS (Defines event)
One person, any skill level but experienced if available. Form P +/- form HI, Pen (waterproof or pencil), Map or GPS if possible. Species ID guide, Phone, Operational procedures, Camera

Then from all animals (carcase condition permitting):

TEAMS 1 and 2

- **Marking, Species ID, Sex, Size/ length and photographs**
- **Blubber thickness** minimal measurements dorsal and ventral in front of dorsal fin.
- **Skin** (genetics)
- **Muscle**

NB IF TOO FEW RESOURCES TO DO ALL ANIMALS take a random sample (allocate numbers to all animals, then randomly select numbers and sample those animals. These samples are essential to define the incident, interpret any additional samples and add to basic knowledge about cetacean populations)
(other samples are relatively quick to collect and allow some genetic, toxicology, dietary (fatty acids and stable isotopes) and endocrinology studies to be carried out)

Then if time/ resources permit:

TEAM 3

- **Teeth, eye and external parasites**

NB IF TOO FEW RESOURCES TO DO ALL ANIMALS take a random sample (allocate numbers to all animals, then randomly select numbers and sample those animals)

Then as time permits

TEAM 4.

- **Full blubber thickness measurements**
- **Reproductive tracts**
- **Remaining internal samples**

At the same time,

- Clinical data should be collected from **live animals** using **the Cl (clinical) form** from the individual animal set.
- If you have an **experienced veterinarian or zoologist** experienced in marine mammal dissection and there are **particular animals showing obvious signs of distress** (eg bleeding ear, obvious injuries, head bashing etc) then these can be dissected, if they die, in more detail using **HI (human interference) and T2-T4 protocols** from the individual animal set.

5.2 ORGANISER

5.2.1 Before you leave

- If you have an established (volunteer) response group, ring all persons on the list and inform them of the stranding, its location and anything that they are required to bring along if they can attend. This will give you a good indication of the number of people you will have to help you.
- Make sure you have the required equipment - best stored for ready collection in bins or something similar. Recommended set-up is for bins for each team and a "marking" bin.

5.2.2 Check list:

- Operational procedure
- Team bins. Location:____
- Marking bin. Location:____
- Main stranding kit. Location:___________________________
- Sample jar bin. Location:____
- Esky and ice for the blubber, muscle and toxicology samples.
  Location:____
- Shovels, bolt cutters and flensing knife.
  Location:____
- 10% buffered formalin and 70% alcohol and other sample preservation equipment
  Location:____

- Spare garbage bins to put all of the samples into and buckets for carrying water, samples and things.
  Location____
- Camera and film if required. Location:___________________________________
- Phones and other communication equipment. Location:______________________
- **You may be required to collect samples for other researchers, check whether or not you do and what extra equipment you may need before leaving- check the updated samples list and contacts.**

5.2.3 When you get to the stranding

- **Please check in with the officer of the state conservation agency. Do not start sampling until you have been given the go-ahead by the person in charge of operations.** You will have to inform them of your actions and identify to them the people involved.

- **Ensure you are covered by a valid state permit for the samples you plan to collect.**

- Number of people required: Ideally fourteen people, but it is possible to manage with any number less than this. Adjustments will need to be made for fewer people by progressively working through the different tasks.

  Team One: Three people required

  Team Two: Three people required (one needs to have experience)

  Team Three: Two people required (one needs to have experience)

  Team Four: At least two people required

  Team Five: Two people required (one needs to have experience)

  Team Six: At least two people required

  If there are time constraints placed on the collection of data and samples and you need to prioritise what is collected please **ensure that the tasks of Teams 1, 2 and 3 are undertaken and completed for all animals.** The tasks of teams 4 and 5 are bonuses and should be undertaken if time allows.

- Each group can be given one of the bins of equipment to do their task – all instructions necessary should be included in each bin. Extra jars, bags, equipment should be available from the sample co-ordinators. **A sharpening stone and steel for sharpening knives is most useful (to be held by the sample co-ordinators).**

- If whales have been partially buried by tidal movement of sand, allocate some people the task of digging the whales out. At the very least, the head, the genital area, a pectoral fin, the dorsal fin and the flukes should be uncovered.

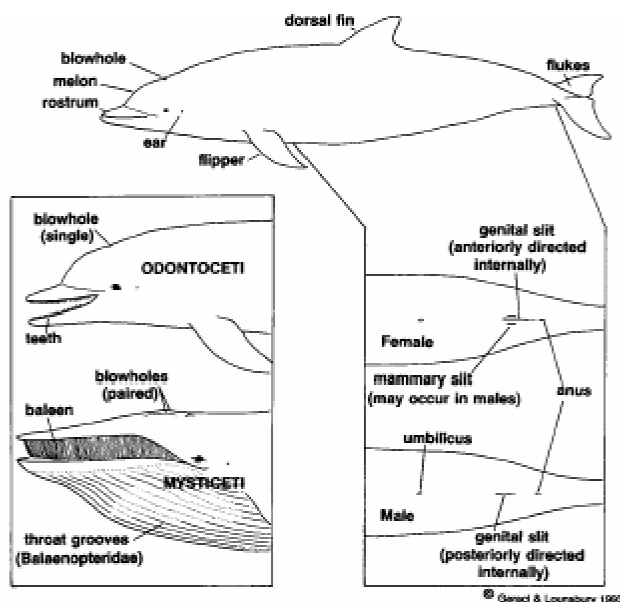
- **Please make sure that all people involved in the necropsies are wearing double gloves – to protect against transmission of diseases/epizootics between humans and marine mammals.**

- You will most likely have to hand in a list of all samples collected to the Incident Controller before leaving the beach. If sampling takes more than one day then you may have to do this on a daily basis. Find out what the Incident Controller requires of you in terms of this before you start sampling so you know what the procedure is.

5.3 TEAM ONE: Marking, sexing and photographs

5.3.1 Marking - three people required.

- This task needs to be done first, as all other tasks need to follow on from this. If there is a surplus of helpers make up another team and get them to start at the other end of the stranding so that this is completed in as quick a time as possible.
- Ensure that Form P is completed. Make sure that an accurate count of the animals is made so that the numbering system is applied to the animals correctly. Numbered cattle tags on cords tied around the tail stock are recommended.
- Number all individuals using the agreed method (see above).
- If possible, roll the animal over onto its side, but only after initial documentation of stranding event has occurred.
- Record the number of the whale in the notebook provided.
- Sex the whale; record this in the notebook. If unsure refer to the diagrams below.



5.3.2 Sexing

- If the whale is female, note if it is lactating (find the teats and make a small incision into the body just posterior to the teats– does any milk exude?), pregnant (is the belly area swollen? run hands over lower abdominal area – can you feel a foetus?) or with calf (is there a calf accompanying?). Record this in the notebook.

5.3.3 Photographing

Photograph each individual. A minimum of three photographs of each individual should be taken:

- one of the genital area to verify the sex;
- one of the tail fluke (try to get the whole of the span);
- one of the whole individual in which the tag number is visible and the individual's pigmentation patterns are clear.
- Photographs should also be taken of any unusual pigmentation, wounds, scars, and external parasites.

- Need to ensure a fail-safe method for recording digital photos and relating them to the individual animal and incident to which they relate.
- For SLR cameras: Record in the notebook the film number and the frame number(s) of photos taken of each individual. When a roll of film is finished, mark the outside of the canister with the roll number, the roll of film with it's number and place it in a snap lock bag with a waterproof label stating the **location of the stranding, the date and the roll number**.
- **Photograph the site.** Photos should be taken of the location of the stranding as well as a full scene of the stranding, so that the topography of the beach can be recorded and the pattern in which the whales came ashore can be noted. **If animals have been moved** from their original positions on the beach prior to you being able to do this please note this down.
- Other teams will mark particular whales with flagging tape where there is something unusual or of significance needed to be photographed. Photograph these as required.

Equipment/Contents of bin

Waterproof notebook	Pens, pencils and textas, datasheets
Rope	Small knife
Film	Gloves

Also need

Gaff, camera, film, cattle tags.

5.4 TEAM TWO: Measurements, Skin, Blubber and Muscle

Three people required, can be done with two.

5.4.1 Measurements

- Measure the animal as instructed on data sheet. Six measurements should be taken and recorded in the sheets provided. Remember, all measurements must be taken in a straight line. **DO NOT FOLLOW THE CURVATURE OF THE ANIMAL**.
- After all measurements have been taken, make an incision along the ventral line of the animal from midway between the pectoral fins to the anus to release any built up gases and stop overheating. Even though the animal is large be careful when doing this **– you don't want to rupture the stomach and for your own sake** you don't want to rupture the intestines!

5.4.2 Skin, Blubber and Muscle

- All skin and blubber samples should be taken from a site just anterior to the dorsal fin. It is important that all samples be taken from here – for comparative studies to be undertaken samples need to be taken from the same location on all animals. Before you start, please soak the cloth provided in the 70% alcohol provided.
- Cut out a block of skin and full thickness blubber 25cm x 25 cm. Place it in a glass jar or Teflon or wrap it in alfoil and put it in a plastic bag. The sample will be trimmed of surface contamination later but keep it as clean as possible.
- Cut out a block of muscle 5cm x 5 cm x 5 cm and place in a plastic bag.
- Use the cloth provided to wipe the blade between sampling each whale – an unclean blade can easily contaminate genetic samples.
- Label with **whale number, carcass code, sex of whale, location and date.**
- Place samples on ice or give to sample runner to put on ice or take to processing station.
- Take a blubber depth measurement at this site. Measure from **the base of the skin to the surface of the muscle** using the vernier callipers provided. Note this in the notebook provided. **Ensure the blubber is not distorted during measurement.**
- Note in the datasheet provided what samples have been collected for each whale.

Equipment/contents of bin

Tape measure Waterproof notebook

Pens, pencils, textas	Waterproof labels
Gloves	Small knife and flensing knife
Flagging tape	Vernier callipers
Aluminium Foil	Snap-lock bags or Teflon bags

Also required: Cloths for wiping down blade, with 70% ethanol;

sample jars, and Esky nearby with ice to hold samples until runner can get them

5.5 TEAM THREE: Teeth, eye and external parasites.

Two (or four working as two teams of two) people required.

5.5.1 Teeth

- Open the whale's mouth and count the number of teeth on the upper and lower jaws. It is sometimes easier to run your fingers along the gum and count the number of teeth, protrusions or bumps that you can feel. Count the upper jaw separately from the lower jaw. Note these numbers in the notebook provided. Count teeth from front to back and enter any missing teeth in sequence ie. 10 (2) 9 (4) is 10 teeth followed by 2 missing, followed by 9 present etc.
- **Please ensure that you document these counts with the number of the whale** – the measurement is useless unless we can reference it to the whale.
- If the animal is a calf note if the teeth have erupted through the gum line and which teeth have erupted through the gum line.

5.5.2 Jaw sample

- Using the bolt cutters or chainsaw provided cut off a section of ONE jaw. A minimum of four teeth must be contained in this sample. If using the bolt-cutters it is sometimes easier to make two slices on either side of the jaw to the bone with a knife. Sit the bolt cutters in these incisions and then cut through the bone.
- Tie a hard tag with the **same number as the whale** around the jaw with the cable ties (or if it is too big for cable ties the string) provided. Place the section of jaw in a snap-lock bag or Hessian-style bag (depending on its size). If using the Hessian-style bag, tie these off with the cable ties provided and attach another hard tag to the cable tie.
- Note the number of teeth on the section of jaw sampled in the notebook provided. **Please ensure that you document this with the number of the whale** – the measurement is useless unless we can reference it to the whale.
- Give the samples to the sample runner or leave the sample at the top (opposite to the direction of the water) of the whale for the sample runner to collect.
- Note any abnormalities and mark the whale with flagging tape so that these can be photographed.

5.5.3 Eye

- Remove an eye (code 2, 3)

- **If you find anything unusual**, mark the animal with flagging tape so that it can be photographed. Once photographed, if applicable take a 6cm x 6 cm x 6 cm sample of the abnormality including some normal and abnormal tissue. If you are unsure check with the sampling co-ordinator. Place the sample in a plastic bag with a waterproof label on which is written **the whale number, carcass code, the sex of the whale, the location, the date and what you have sampled. Mark the sample "LESION" and put the sample on ice with the others.**

5.5.4 External Parasites:

- Check the whole of the external surface of each whale for external parasites. Check the blowhole, the mouth and any orifices for parasites
- If there are any present, **mark the whale with flagging tape** so that the photographer knows to photograph it. After it has been photographed, remove the parasite (use a knife to cut it out if it is partially buried) and place in a sample jar.
- Label the inside of the jar with a waterproof label on which is written the location, date, the number of the whale, the sex of the whale and what the sample is.
- Label the outside of the jar with the same information using an indelible marker.
- Give the sample to the sample runner or leave the sample at the top (opposite to the direction of the water) of the whale for the sample runner to collect.

Equipment/contents of bin

Waterproof notebook	Pens, pencils, textas
Waterproof labels	Knife
Aluminium foil	Snap-lock bags or Teflon bags
Gloves	Vernier callipers
Flagging tape	Cloths for wiping down blade, 70% alcohol
Hessian-style bags	Hard tags and string or cable ties

Also required: bolt cutters and nearby esky with ice to hold samples till runner can get them.

5.6 TEAM FOUR: Full blubber thickness, Reproductive Tracts and Remaining Internal samples

At least two people required.

- From the ventral incision, cut transverse sections up along the flanks of the animal at 4 points - see data sheet (if it's easier to do more – do so!): just behind the ribs, in line with the anus and midway.
- Collect the series of blubber measurements
- Pull back the blubber and muscle (or cut it away), revealing the body cavity.
- You may have to cut the ribs to gain access to the body cavity – the easiest way to do this is with the bolt cutters. Have a look over the body cavity and the organs contained in it. Note whether any blood has pooled in the body cavity. Write this down in the notebook provided. **Please ensure that you document this with the number of the whale** – the measurement is useless unless we can reference it to the whale.
- You should be able to see the lower portion of the lungs, the liver (big reddy/brown organ) sitting over the upper part of the stomach, the stomach and the intestines. Note the colour of the organs, whether any appear to be abnormal, swollen, unusually small, an odd colour etc. Note whether the lungs have froth on their external surface (indicates water in the airways). If any abnormalities are found mark the animal with flagging tape so that these can be photographed.
- Search through the body cavity and over the organs for any parasites. Collect any found. Put these in individual sample jars of fresh water with a waterproof label on which is written **the whale number, the sex of the whale, the location, the date and what you have sampled.** Write the same on the outside of the jar with an indelible texta.

5.6.1 Liver, kidney, spleen and heart blood samples (code 2, 3)

Take a sample of the kidney and the liver. Refer to the diagram (to be obtained) provided if you're having difficulty finding them. The liver is bi-lobed (made up of two large lobes), is a browny-red colour and a smooth external surface. It's usually the large organ lying over the top of everything else. The kidney lies further towards the posterior end of the body cavity and lies near the dorsal surface of the cavity. It is usually a browny-purpley colour and has a lumpy appearance, being made up of a lot of nodes.

- These samples need to be about 500g and should be taken from the caudal part of the organ.
- Place each in a snap-lock bag with a waterproof label on which is written **the whale number, carcass code, the sex of the whale, the location, the date and what you have sampled.**
- **Aspirate about 10- 20ml of blood from the heart or other large vessel using a syringe.**
- **Place on ice.**

5.6.2 Reproductive Organs (code 2,3)

The reproductive organs are located at the very posterior of the body cavity. The easiest way to find them is to locate the genital slit and search around the dorsal area of body cavity above this. Dissect out both ovaries and testes. If you are able to locate the uterus, dissect this out. Put each into a sample jar or plastic bag with a waterproof label on which is written **the whale number, the sex of the whale, the location, the date and whether the organ is the left or the right ovary or testis**. Write the same on the outside of the jar with an indelible texta. Keep cool.

- Also collect a sample of the mammary gland. These are located directly above the mammary slits. Place this in a sample jar with a waterproof label on which is written **the whale number, the sex of the whale, the location, the date and whether the sample is from the left or the right mammary gland.** Write the same on the outside of the jar with an indelible texta. Please note if the mammary gland contains milk.
- If there is a foetus present, mark the whale with flagging tape so that this can be photographed. Remove the foetus if it is small enough. Measure it's total length (tip of upper jaw to the deepest part of the fluke notch) and place in a sample bag (either a snap-lock bag or a garbage bag) with a waterproof label on which is written **the number of the mother, the sex of the foetus, the location and the date.** Note this in the notebook provided. If the foetus is too large to collect please ensure that a total length is taken and the foetus is photographed.

Note all samples collected in the notebook provided. **Please ensure that you document this with the number of the whale**.

5.6.3 Stomach samples (code 2,3,4)

It may be easier to remove the stomach before sampling the liver and the kidney. Just be careful not to cut out these organs as you dissect out the stomach.

- Feel around the stomach until you have located the upper part where it connects with the oesophagus. Tie off the oesophagus with some string above where it joins the stomach. Again feel around the stomach until you have found the lower part where it connects with the intestine. Tie off the intestine below where it joins the stomach. Cut the oesophagus **above** where you have tied it off and cut the intestine **below** where you have tied it off. Pull the stomach out of the body cavity – you may have to cut it away from its connective tissue and slide it into a garbage bag with a waterproof label on which is written **the whale number, the sex of the whale, the location and the date**. You may find that you need to put it into several garbage bags – it's quite a bloody job.
- If the stomach is too large to remove from the body cavity, cut it open (make a cut along the longer surface of the organ) and scoop the contents (if there are any) into the sieve provided, putting a handful aside chilled in a plastic bag if the animal is code 2 (for biotoxins). Using one of the buckets provided wash the main sample through with seawater and place in a garbage bag. Make sure that the bag contains a waterproof label on which is written **the whale number, the sex of the whale, the location and the date**.
- Note in the notebook provided whether the stomach sample is (A) the complete stomach, (M) most of the contents of the stomach (>75%) or (P) part of the contents of the stomach (<75%). Note if the stomach was empty and therefore no contents were collected. Also if calves have milk in their stomachs please note this.

5.6.4 Pathology

(Form T4) Histopathology/ histoanatomy samples(code 2,3), Swabs (Code 2), Photos (Code 2-4)

Veterinary expertise necessary. Collect 15mm thick slices (one from the middle and one from the edge) of each of liver, kidney, spleen, lung, heart wall, adrenals, thyroid (bilobed, ventral at base of trachea), thymus if present (chest cavity in front of heart), mesenteric lymph nodes (where intestines attach to dorsal abdominal wall, and thoracic lymph nodes,

brain if available and any potential abnormalities (sample to bridge normal and abnormal tissue). Keep chilled until placed in 10x volume of 10% formalin.

Equipment/contents of bin

Waterproof notebook	Pens, pencils, textas
Gloves	Small knife
Large knife	Snap-lock bags
Garbage bags	String
Waterproof labels	Blubber hooks
Sieve	

Also required: sample jars, bucket for water to rinse gut samples

5.7 TEAM FIVE: Sample Preservation, Shipping, Tracking (incl forms)

Samples

Two people required. Whilst not involved in dealing with bodies on the beach, this is one of the **most important** tasks.

- Firstly make sure you know where all of the extra bits and pieces are so that when samplers need extra gloves, pencils, sample bags etc. you can provide them quickly.
- Organise the garbage bins for each type of sample, e.g. stomachs, teeth, livers etc. If there are a large number of animals you may need multiple garbage bins for each type of sample.
- Organise the eskies so that they have ice in them. If there are a large number of animals you may have to fill a couple of the garbage bins with ice.
- Organise the 10% buffered formalin and 70% alcohol and AFA so that when samples come in you can fill the sample jars with the appropriate preservative.
- One person will act as the runner, collecting samples from the beach and returning them to the sample storage point. The other will (i) check that all samples coming in have **the number of the adult, carcass code, the sex of the foetus, the location and the date** on a waterproof label inside the jar/snap-lock bag and also on the outside of the jar/bag; (ii) preserve each sample in the appropriate manner; (iii) place each sample in its appropriate bin.
- For treatment of samples follow protocol at Section 1.10 (Processing of Samples). A judgment will need to be made whether to process all samples on site or to keep them chilled for transport to a better facility with more personnel. This will depend upon: facilities at the site, number of animals to be processed, the quality of their carcasses (therefore the number and fragility of samples) and the number of available personnel. Refer to Appendix 1 - **SUMMARY PRESERVATION, SHIPPING and TRACKING Form**. If samples cannot be processed properly within the correct time – consider not collecting them.

Please take care when handling the preservatives. Formalin and the contents of the skin sample vials are toxic. Always wear gloves, do not get any of the preservatives on your skin or inhale them. If you do get some preservative on your skin please

wash it off straight away. If there is a substantial spill of any of the preservatives please inform the organiser.

Make sure all bags are sealed properly, all jars have their lids on properly.

- Note in the datasheets provided all the samples that are collected as they come in. **Please ensure that you document this with the number of the whale**.

Equipment/contents of bin

Waterproof notebook	Pens, pencils, textas
Waterproof labels	Gloves
Snap-lock bags	Garbage bags
Tissues, paper towel	

Extra equipment (some of this may be in main kits):

Garbage bins	Buckets
Sample jars	10% buffered formalin
70% alcohol	Eskies
Ice	Knives
Tape measure	Sharpening steel and stone
Sunblock	Water
Pipettes	Vials (eppendorfs etc for serum etc)
Teflon bags	Sterile scalpel blades
Microbiology swabs	Cigarette lighter

5.9.1 SHIPPING INFORMATION

Additional information is contained within the IATA regulations for shipping dangerous goods. The following is a guide only. You should check with the relevant shipping/ customs/ environmental/ quarantine agents or sample recipients for the relevant permitting/ packaging and storage requirements of your state or agent. (NB: IATA approved shipping containers can be purchased)

- Make arrangements for samples to be maintained at storage temperature during shipping. Especially avoid freeze/ thaw.
- Ensure the recipient will be there to receive the samples
- For samples in liquid preservative (eg formaldehyde), preserve for approx 1 week then remove the liquid and wrap sample in gauze swab or cloth moistened with the preservative and seal container well.
- Double-bag samples with a label between (paper tag, pencil) and outside (lab marker) the bags.
- Place in a robust container (with dry ice if appropriate).
- Place in Styrofoam packed box or esky with appropriate coolant.
- **In general, sample must be within 2 sealed, impact resistant containers with sufficient absorbent material to absorb all liquid enclosed in case of leakage.**
- Enclose copies of relevant data forms. (**keep a copy**)
- Complete tracking form and enclose a copy (**keep a copy**)
- Ensure copies of relevant permits are attached on the **outside** of the package for inspection by authorities

64 **Limited classes of dangerous goods which may be carried (Australia Post)**

64.1 Dangerous goods specified in clauses 64.2 to 64.3 inclusive, may be lodged and carried by post providing they are properly packed and comply with such terms and conditions governing their carriage.

64.2 **Infectious perishable biological substance** which are dangerous goods may be lodged and carried by post within Australia providing:

64.2.1 an article containing such a substance is:

(i) addressed to a recognised laboratory, hospital, clinic or regulatory body;

(ii) lodged at an office for delivery within Australia by:-

1 a qualified medical practitioner or veterinary surgeon;

2 hospital, clinic, regulatory body or recognised laboratory;

3 a member of a Commonwealth, State or Territory police force; or

4 a person who has the authority of the agencies in clause (ii) above;

(iii) sent at the perishable infectious biological substances rates determined by Australia Post; and

(iv) packaged and presented in the manner prescribed in the current Technical Instructions of the Civil Aviation Organisation as reflected in the IATA Dangerous Goods Regulations;

64.3 **Non-infectious perishable biological substances and solid carbon dioxide (dry ice)**, when used as a refrigerant, may be lodged and carried by post within Australia providing:

64.3.1 an article containing such a substance is:

(i) addressed to a recognised laboratory, hospital, clinic or regulatory body;

(ii) lodged at an office by:-

1 a qualified medical practitioner or veterinary surgeon;

2 hospital, clinic, regulatory body or recognised laboratory;

3 a member of a Commonwealth, State or Territory police force; or

4 a person who has the authority of the agencies in clause (ii) above; and

(iii) packaged and presented in the manner prescribed in the current Technical Instructions of the Civil Aviation Organisation as reflected in the IATA Dangerous Goods Regulations.

6 ADDITIONAL NOTES ON RESEARCH AND REVIEW OF BEST PRACTICE SAMPLING METHODS

This document is a first step in providing workers with some additional information on sampling and sample preservation. Some alternatives to methods in the protocols are included as is some basic information on the reasons for collecting samples in the methods prescribed.

6.1 Life history and basic stranding information

6.1.1 Introduction

The life history information such as stranding information, species, age, sex and reproductive status are essential to documentation of the event and interpretation of all other samples and should be collected on as many animals as possible.

In addition to gross morphological sampling, material for genetic studies can be easily collected from live, freshly dead or decomposed animals and can provide information on speciation, population structures and social organisation within the stranded group. "Details such as colour patterns, external measurements, sex, an estimate of age and reproductive state and tooth counts are important and can be obtained from both live animals and carcases. In addition the whole skeleton from carcases can help in resolving new genotypes that might be found. The reverse is also true, that genetic analysis is important in resolving differences based on bone structure. If the skeleton can't be transported from the stranding site to a storage facility or burial site then it should be buried at a precise location near the site of stranding, for later retrieval". (Hale 1996)

6.1.2 Stranding information

Should be recorded on a standard form, designed for easy entry of data to a database. A national standard would facilitate access to data for epidemiological studies and tracing of archived samples.

6.1.3 Morphometry

- Standardised measurements are essential and any deviations should be noted. The most important measurement to take is a straight-line total length from tip of rostrum to the deepest part of the notch in the tail fluke;
- Photographs taken squarely at right angles to the animal to record features, injuries, colour patterns. Good quality slide film is preferable to print film and digital photography facilitates distribution of images to experienced people for comment; if no photographic equipment is available, use sketches; preferably use both photographs and sketches.

6.1.4 Skeletal anatomy

- skull or an identifiable part of skeleton/teeth of rare or hard to identify species (Jervis). Entire skeletons of some species for museum collections. The South

Australian Museum are leaders in this area in Australia and we have drawn on their protocols and priority lists for this report (Kemper Unpublished).

6.1.5 Genetics

Muscle is preferred if the carcass is less than 24h old, otherwise skin is better as it dries first and therefore degenerates less (Hale 1996).

- $5mm^3$ in small strips for fixation if fresh, 4 times that if not(Hale 1996, Rowles et al. 2001).
- A 10x vol of saturated salt in 5-20% DMSO in water (DMSO, Sigma Chemical Co., Cat: D8779) is the preferred fixative (Hale 1996, Rowles et al. 2001) also (Duffield et al 2001). Saturated salt can be used without DMSO if necessary. Ethanol 80% is recommended by some (Rowles et al 2001; Duffield et al 2001) but is considered to be third choice by (Hale 1996). Samples can also be frozen (Duffield et al 2001). **Saturated salt solution is a solution where no more salt can be dissolved in it.**

"With skin it's important to sample the right thing. Cetacean skin consists of a fine, paper thin, outer layer of dead cells (epidermis) overlying a 2-8mm layer (the dermis, which may be dark or light in colour) of live cells, under which is the thick white connective tissue layer. This layer may contain fat, depending on the place on the body and the physiology of the animal. It is the live cell layer of skin that is best for the extraction of DNA. The fatty connective tissue should be trimmed from samples taken from dead animals. Skin samples for DNA extraction can be obtained from live stranded cetaceans by scraping the skin to a depth of 1.5-3 mm. This ensures that live cells are sampled. A veterinary biopsy punch held at a low angle to the skin, a scalpel used very gingerly or a "cheese grater" built for the purpose can be used to obtain the sample." (Hale 1996)

- Blood is best collected in EDTA, but heparin can be used if necessary. It should be chilled (not frozen) until it can be centrifuged and the buffy coat (white cells at the interface of the red cells and plasma) aspirated and frozen below -80°C in no more than 0.5 - 1.0 ml of liquid / 10ml of whole blood. (Hale 1996).

6.1.6 Age

6-8 teeth from the mid- left mandible (roots intact), ear bones of baleen whales (frozen, 70% ethanol, dried);

- baleen of mysticetes (dried)
- earplugs of mysticetes (10% formalin);
- eye lens (frozen: freshly dead animals/ code 2 only);
- frozen flipper for growth plate radiography (Kemper Unpublished, Rowles et al. 2001).

6.1.7 Reproduction

determine sex of animal: if female determine if lactating/ pregnant;

- Blubber, faeces, urine, serum (frozen at -20°C) for hormone analysis (primarily for validating methods used on live animals and providing additional information on animals in the absence of internal examination).
- Mammary development, milk samples;
- collect both gonads- weigh and measure (Testis- weigh and measure testis and epididymis separately; Ovaries-examine for CL/ follicles). Fix in 10% formalin;
- examine the tract for signs of a current or previous foetus. Measure uterine wall thickness, cervix diameter, and internal diameter and length of uterine horns. Fix in 10% formalin. (Rowles et al. 2001)
- If testis or uterus are very large, fix a cross- and longitudinal section of the testis (Rowles et al. 2001)and representative portions of the uterus (vagina, cervix, uterine horn, fallopian tube)

6.2 Health, disease and clinical assessment

6.2.1 Introduction

(Miller et al. 2001) reviewed emerging diseases in marine mammals: Cetaceans are host to a range of emerging viral, bacterial and neoplastic diseases. Three distinct, but not necessarily species- specific morbilli viruses have been described in cetaceans (dolphin morbilli virus, porpoise morbilli virus, pilot whale morbilli virus) and another, unknown morbilli virus has been detected in a fin whale (Balaenoptera physalis). Morbilli viruses have emerged as significant pathogens worldwide but their pathogenesis and epidemiology are only partly understood. Benign mucosal and cutaneous papillomas and fibropapillomas, associated with a papilloma virus, have been described in a range of odontocetes and there is unpublished data describing arboviral encephalitis in one Orca, and hepadnaviral hepatitis in two species of dolphin. Brucella spp have been isolated from a range of odontoctes and mysticetes. These bacteria have potential economic and public health significance. A novel species of helicobacter has recently been described in association with ulcerative gastritis in dolphins. This review also mentions Lobomycosis, Histoplasmosis, Coccidioidomycosis, immunoblastic malignant lymphoma, squamous cell carcinoma, renal adenoma, pulmonary carcinoma, and angiomatosis which have been described in various species of dolphin since 1997.
Gulland et al (2001) identifies a massive range of diseases for which no infectious cause has yet been identified, such as neoplastic, degenerative, and congenital defects, most of which unknown pathogenesis, aetiology or significance. Amongst these, the prevalence of neoplasias appears to be increasing but it is not clear whether this is a true increase or due to improved surveillance.
Thus it is clear that a wide range of diseases of cetaceans are of unknown cause and of unknown ecological, economic and public health/ OH&S significance. Aside from the potential of large, long- lived animals for monitoring environmental toxins, the impact of these toxins on the animals are unknown. The significance of diseases, clinical observation and blood parameters to the survival of released cetaceans are also largely unknown. Study in these areas will assist our management of stranded cetaceans and our interpretation of findings in other fields.

6.3 Clinical pathology and physiology

6.3.1 Introduction

Clinical pathology specimens are generally those that can be collected from living animals. Some, such as serial blood samples and clinical examination data, when validated against post-release observation or necropsy material, have the potential to provide us with prognostic indicators to assist in our management of live stranded animals and as such are of high priority. Other samples allow us to obtain information from living animals, on health, disease and the environment that is not confounded by terminal physiological events or decomposition.

6.3.2 Bacteriology

Samples for bacteriology must remain uncontaminated from bacteria from the environment or other sites within the animal and there fore should be collected as soon as possible after death (especially in the case of sites adjacent to or with a close circulatory association with the digestive tract, such as the liver) and as soon as possible after tissues have been exposed during necropsy. Apart from lesions, tissues commonly sampled include lymph node, lung, spleen, kidney and liver as circulating bacteria commonly lodge within these organs.

- Tissues and lesions: Ideally, $6cm^3$ pieces of tissue should be transported chilled to a clean environment, the surface sterilised by searing or by swabbing with ethanol or 10% formalin and allowing it to air dry, and then the swab placed into an incision made through the sterile surface using a sterile scalpel.

- Fluid (abdominal, pericardial, thoracic fluid; blood) is best aspirated using a needle and syringe and then transferred to the lab chilled. If the lab is more than 48 hours away, fluid should be transferred to a swab in transport media.
- Swabs should be transported to the lab in Amies or Stuarts transport media at 20-25°C (Dunn et al 2001) or chilled (Geraci and Lounsbury 1993).

- If tissue swabs or fluids cannot be cultured within 72 hours, tissue or fluid samples can be stored at -70°C.

6.3.3 Virology

- Padraig Duignan, Massey University has a protocol for collection of samples for Morbilli virus.
- CRC and Geraci recommend - lung, liver kidney, spleen, LN and brain- 6 cm^3 pieces frozen at -20C or -70C (for isolation and PCR) and in addition Geraci and Lounsbury (1993) recommends serum, urine and pericardial, pleural and peritoneal fluids.
- CRC recommends if within 6 hrs and have cooling fix $1mm^3$ in glutaraldehyde 3-5% (EM), otherwise formalin fixation/ snap-freeze for immunohistochemistry.
- For serology, try to get serial samples during rehabilitation .p285
- Tissues for viral isolation can also be stored short term, chilled in 1-2 ml saline with 5%BSA, 50ug/ml gentamicin.

6.3.4 Parasitology

(Geraci and Lounsbury 1993) and (Duignan 2000) both recommend preserving endoparasites in AFA (100ml formaldehyde 37%, 400ml distilled water, 500ml ethanol, 20 ml glacial acetic acid) for morphology and in 70% ethanol for genetic study. Faeces can be fixed in hot 10% formalin then transferred later to 70% ethanol and ectoparasites can be stored in 5% glycerine in 70% ethanol (Geraci and Lounsbury 1993). Lesions with associated parasites should be fixed in 10% formalin for histopathology.

As alternatives, endoparasites can be placed in glacial acetic acid for 10 min then ethanol or fixed in 5% formalin (Duignan 2000) or nematodes in 10% formal saline or 70% ethanol and cestodes and trematodes relaxed in fresh water and then placed in 10% formal saline (Warneke 1997).

For morphology, at least 5 of each sex are needed so as many as possible should be collected.

6.3.5 Haematology

At least 1 mL blood should be placed in EDTA (first choice) or Heparinised tube and rocked back and forth to mix the anticoagulant. Inspect to ensure there are no clots, transport at 4°C, to be processed at the lab within 48 hours. Ideally air-dried thin smears should be made from fresh or recently anticoagulated blood. Smears are made by:

- Place 3 x 1.5mm diameter drops of blood at one end of a slide,
- Stand a second slide on its end and place it in the middle of the first slide
- Slope it back towards the blood drops at about 45 degrees and then drag it up the slide till it contacts the blood drops and the blood runs along its edge
- Push the slide along, dragging the blood behind it. This should produce a very thin but continuous smear. The action must be smooth. Adjust the speed and angle of the slide and the amount of blood till a smear of one cell thickness is achieved.

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6.3.6 Biochemistry

Biochemical analysis requires at least 1 mL of plasma (the clear portion after 3 mL of heparinised blood has been centrifuged at 3000 rpm for 10 min). As a second option, if fibrinogen is not to be measured, serum can be used (the clear portion after clotted blood has been centrifuged at 3000rpm for 10 min). For glucose measurement, 1 mL of blood should be collected in a Fluoride Oxalate tube as this stops the red cells from metabolizing glucose and thereby reducing the blood glucose concentration during transport.

6.3.7 Endocrinology

Serum, frozen at -20°C or less. Anticoagulants interfere with some assays. Note the time of collection as some hormones have circadian periodicity (St Aubin 2001).
Saliva- More work needs to be done to validate. Caroline Hogg, Australian Marine Mammal Research Centre is working on this (2004).

Faeces and blubber are better suited to some studies as their hormone concentrations are less periodic than in plasma but further work is required to validate their use in cetaceans. Stored frozen at -20°C or less.

6.3.8 Immunology

6.3.8.1 Serological

Serum stored in 1 mL aliquots at -70°C (short- term -20°C is acceptable). Plasma can be used for some studies if serum is not available but produces higher background levels in some tests. Parameters that could be measured include specific and non- specific indicators of disease or exposure to pathogens. These include total and individual immunoglobulins, specific antibodies, antigens and acute phase reactants.

6.3.8.2 Cellular

The study of cellular immunity in wild species is a new and developing field. A wide range of techniques (e.g. measurement of lymphocyte subset ratios by antibody labelling, various leukocyte stimulation assays), are being tried in various species, therefore there is no universal sample collection technique. The collection of samples for this work should be for specific projects that supply their own protocol.

6.3.8.3 Toxicology

6.3.8.4 Heavy metals

(Duignan 2000) recommends liver/ kidney, sometimes bone (lead) or muscle, stored in plastic or glass with teflon seals. The composition of the knife blade should be noted (titanium preferred but often not practical).

(O'Hara and O'Shea 2001) recommends at least 20g of liver (left caudal lobe), kidney (left), skin from lateral wall, and whole blood from animals of carcass code 1-3, tissues collected using a stainless steel knife or scalpel blade.

Inductively coupled plasma mass spectroscopy, though expensive, allows the use of small quantities of tissue and may become more prevalent in the future (1 g is sufficient for a full range of metals).

6.3.8.5 Organochlorines

From animals of carcass codes 1-3: 100g (400g for archive) blubber (full thickness), liver (left caudal lobe), and brain. At least 6 mL blood. Frozen in clean glass or Teflon (O'Hara and O'Shea 2001).

6.3.8.6 Polyaromatic hydrocarbons

From class 1 and early class 2 animals only: 5 mL bile from hepatic duct, 50g liver, and blood. Frozen in liquid N, protected from light (O'Hara and O'Shea 2001).

6.3.8.7 Biotoxins

(Duignan 2000) recommends collecting the following samples. The first two are similar to those suggested by (O'Hara and O'Shea 2001):

- 5-10 mL frozen plasma/ serum
- 100g each liver/ kid/ brain/ milk/ blubber (use ethanol-washed stainless steel knives and store in plastic bags and freeze)
- one 70ml container of stomach contents and freeze, one 70ml container 3/4 stomach contents rest ethanol
- 300ml sea water mixed with 100ml lugols iodine

- for some toxins, fixed brain (amnesic shellfish poisoning) and respiratory or mucosal tissues (neurological shellfish poisoning) can be used for immunohistochemical staining. (O'Hara and O'Shea 2001).

6.3.8.8 Mixed function oxygenases

Mixed function oxidases are inducible enzymes that are used to metabolise a range of compounds. Induction in response to exposure to toxins may be used as a marker of exposure to a significant load. Induction of MFOs also leads to increased rate of breakdown of endogenous substances eg hormones so results may assist with interpreting some endocrinological or pathological findings. They are rapidly broken down and therefore must be sampled (from skin/ liver) and placed in liquid N within 1-2 hrs of death

6.3.8.9 General

The four main issues with the collection of material for toxin analysis are contamination, consistency of sample site, storage space, expense of analysis, and quality control of analysis. The latter three will be addressed in more detail later in the accompanying issues paper but in summary, there is need for a study to determine the effect of decomposition on results, to confirm which animals it is worth collecting these materials from (Thiele 1996) and attention should also be paid to establishing a collection of samples for use as quality control standards as results are likely to vary between labs and with different techniques. Quality control, and adequate funding, may permit ongoing analysis of samples and reduce the amount of archival space required. As many toxins are cumulative and affected by events such as lactation, good life history data is essential to the interpretation of findings whether the information is to be used for health studies or as an indicator of environmental contamination. In addition, comprehensive pathology data is useful to interpret the significance of different levels to the animal.

Contamination is best avoided by using the correct knives and storage vessels and by trimming of samples in a clean environment with decontaminated implements. The NMMTB archive in the USA is the gold standard for archival material and recommend collection from code 2 animals only, 400g each of liver, kidney and blubber in teflon bags, trimmed with a titanium knife and stored below - 80C (preferably liquid N). In addition, 6ml of blood should be collected (Becker et al. 1994).

In reality, titanium knives are easily dulled and often not available, so most use sterile high quality stainless steel blades and record the blade type that was used (metals from the stainless steel can contaminate the sample).

As a compromise, for general purposes, (Geraci and Lounsbury 1993) recommends the following samples be refrigerated for the short term then trimmed as soon as possible and frozen below -20C (preferably -70C):

- organics- blubber and liver in Teflon, Alfoil or borosilicate glass
- inorganics (such as metals)- liver and kidney in Teflon, polyethylene or borosilicate glass
- biotoxins- blubber, liver and stomach contents

In Australian references (Warneke 1997) recommends at least 50g liver, kidney, muscle, blubber and brain and at least 100g of each for pesticides and metals. (Thiele 1996) and Evans (unpublished) recommend 5mmx 5mm x full depth skin and blubber and (Kemper

Unpublished) recommends two 100g bags each of kidney, liver, blubber (priority) and muscle. Sample sites in Australia vary between the mid-lateral aspect of either side of the body, at the level of the dorsal fin (Geraci and Lounsbury 1993, Kemper Unpublished) and a dorsal site anterior to the dorsal fin (Evans, unpublished) for blubber and the caudal pole of the kidney (Geraci and Lounsbury 1993, Kemper Unpublished) and the middle of the kidney (Evans, unpublished). Blubber is not homogenous (Koopman et al. 1996; Best et al. 2003; Evans et al. 2003; Evans et al. 2004).

6.4 Anatomy and anatomical pathology

6.4.1 Histo-anatomy and histo-pathology

Tissue pieces no thicker than 10mm should be placed in 10x volume of 10% neutral buffered formalin and kept at room temperature. The jar should be labelled inside (pencil on cardboard) and out. To optimise the potential for samples to be used for retrospective immunohistochemistry studies at a later date, it is preferable that the tissues are embedded in paraffin as soon as the samples are fixed (approx 48h).
For pathology examination, if time and facilities are short, often only lesions are collected but the interpretation of these lesions is assisted greatly by the examination of a whole suite of tissues which commonly show only microscopic but nonetheless important changes. Normally a compromise must be reached. The entire range of samples comprise:

- lung, trachea, heart, aorta, pulmonary artery, thymus, salivary gland, thyroid, tonsil, tongue, gastro-intestinal tract (oesophagus, stomach, duodenum, jejunum, ilium, colon), pancreas, spleen, liver, adrenal, kidney, ureter, bladder, urethra, gonad, prostate, uterus, penis, eye, brain, spine, marrow, muscle, skin, blubber
- lymph nodes (submandibular, cranial cervical, prescapular, axill, tracheobronchial, hilar, gastric, hepatic, mesenteric, colonic, sub lumbar, inguinal).

6.4.2 Immunohistochemistry and In-situ Hybridization

These techniques involve the labelling of specific antigens (immunohistochemistry) or DNA (in-situ hybridization) allowing the visualization of micro-organisms, anatomical structures, or proteins (eg some toxins) in tissue.
Formalin fixation is suitable for most procedures, especially if the tissues are in formalin for only a few days before embedding in paraffin. More fragile targets sometimes require that tissues be frozen; however, freezing compromises tissue morphology.

6.4.3 Acoustic pathology

The correct removal of ears is difficult and requires some training. Dr Darlene Ketten has designed a protocol for this purpose which can be obtained from DEH on request. **NB**: alcohol dissolves otoliths – they should be stored dry.

6.5 Forage ecology

6.5.1 Stomach contents, faeces

Formalin dissolves otoliths, therefore they should be washed with saline and conserved frozen or fixed in alcohol. Freezing destroys plant matter and algae, therefore they should be preserved in alcohol or formalin. (Rowles et al. 2001).

6.5.2 Blubber for fatty acids

1cm x 1cm x full thickness- Location is important. Stored frozen.

6.5.3 Stable isotopes

Skin- 3cmx 3cm
Plasma 1.5ml
Liver/ Kidney/ spleen/ muscle 1 cm x 1 cm x 3 cm
Bone- need to decide upon a standard position

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