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Epidemiology, disease, and health assessment

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7.1 Introduction

Understanding marine mammal health and disease and the related impacts on populations is crucial to support effective conservation and management decisions. However, ethical issues involved in conducting experimental studies can limit the scope of marine mammal health research. This forces a focus and reliance on epidemiological studies, similar to those that have been applied to studying factors affecting human health. Marine mammal epidemiology is additionally challenging because most marine mammals are not easily observed for most of their lives, disease states are generally difficult to detect, and reporting mechanisms for disease used in human and veterinary epidemiology (i.e. birth, death, and disease records) are virtually non-existent for marine mammals. Nonetheless, despite these drawbacks, there are many ways in which robust and reliable epidemiological studies can be applied in the field of marine mammal science.

Marine mammal health and disease issues are gaining global attention and a coordinated approach to their study, such as using standard protocols, will enable meta-analyses (combining information from multiple studies) to be carried out in the future. It will then be much more feasible to identify the critical hazards for marine mammals, particularly if the well-defined and accepted epidemiological approaches outlined here are utilized. Advances in capture and sampling methods (see Chapter 2), the expansion of stranding surveillance networks for marine mammals, the centralization of stranding records (Gulland *et al.* 2001a), and follow-up monitoring of mortality and morbidity using direct observation (Wells and Scott 1990; Gulland 1999), all now give us an unprecedented opportunity to adapt methods from human and veterinary medicine. Necropsies of stranded and by-caught animals, as well as visual assessment, remote biopsy, and capture–release

Box 7.1 Definitions of useful terminology

Exposure: Disease-causing factors, including infectious, toxic, nutritional, traumatic, genetic, degenerative, physiological, social, and behavioural.
Confounding factor: A factor or variable that correlates with both the exposure and response (i.e. independent and dependent variables in statistical terminology) so that it masks an actual association or falsely indicates an apparent association.
Incidence rate (IR): The number of new cases (disease onsets) divided by the sum of the time over which the individual animals were observed.
Cumulative incidence (CI risk): The proportion of individuals free from disease which *develop* a specific disease over a specified period.
Incidence odds ratio: The ratio of the number of individuals that experience the disease to the number who do not.
Prevalence: The proportion of a population which *has* a disease at a particular point in time.
Cohort: Populations or groups within a single population that are followed over time.

health assessment of free-ranging individuals, provide information on causes of death, endemic diseases, emerging diseases, and toxin exposure. This information can then be related to trends in the physical, chemical, and biological environment.

Epidemiology[1] is defined as the study of the distribution and determinants of health-related states in populations (Last *et al.* 2000). As such, it focuses on examining the *occurrence* of disease with the premise that disease does not occur randomly. It is concerned with impacts on populations not individuals. Over the last 50 years or so epidemiological science has evolved into two distinct disciplines that are relevant to wildlife: modern or causational epidemiology (Rothman and Greenland 2005) and infectious disease epidemiology (Hudson *et al.* 2002). Causational epidemiology is the scientific method for investigating potential causal links between exposures and responses. Exposures do not become causes until there is sufficient evidence for a causal link between the agent and the specific response or health state of interest. Additionally, a set of clearly defined study designs to test for causal links have been developed and refined since at least the 1960s.

By contrast, infectious disease epidemiology is concerned with determining the impact that an outbreak of infection (i.e. viral, bacterial, parasitic, or fungal) will have on the dynamics of the host population. Although there is much overlap between these two branches of epidemiology, particularly when investigating the role of confounding variables on disease occurrence, infectious disease

[1] Strictly speaking the term epizootiology should be used to refer to diseases in animal populations. However, the word epidemiology is now widely applied to studies of human, veterinary, and wildlife diseases.

epidemiology has largely evolved as a branch of mathematical and statistical modelling (Grenfell and Dobson 1995). Since the causal agent is known, the focus is then to predict the likely spread of infection during an epidemic outbreak, to estimate the cycle of infection and its inter-epidemic interval, and to investigate the potential impact of intervention measures such as vaccination.

In this chapter we will be largely concerned with the science of causational epidemiology. It is our intention to outline the principles and practices of causational epidemiology as it applies to marine mammal science. Additional information can be found in the extensive medical and veterinary epidemiological literature.

7.1.1 Exposures and responses

Epidemiology is premised on the observation that disease has different causal factors that can be determined by comparing disease rates in different populations, or groups of individuals, that vary with respect to their exposures. It is these exposures that are the putative causal factors for a given disease. The term exposure covers a wide range of causal factors: infectious, toxic, nutritional, traumatic, genetic, degenerative, physiological, as well as social and behavioural causes. In some cases the agents or exposures responsible for the specific disease are fairly obvious, as is often the case where infectious agents or toxins are involved. However, for marine mammals the factors involved are not always as apparent as they might be among terrestrial or domestic animals, largely due to the relative inaccessibility of many species.

Although the presence of a particular infectious agent or toxin is 'necessary' for a specific disease to occur, even these diseases are not usually caused by a single factor and additional causal or risk factors will be involved (Rothman and Greenland 2005). The most widespread causes of disease identified to date in marine mammals are certainly infectious, resulting from their exposure to arthropods, helminths, protozoa, fungi, bacteria, and viruses (Dierauf and Gulland 2001; Gulland and Hall 2007). Typically, epidemics have been associated with viral and bacterial infections, whereas the larger infectious agents (such as the helminths) more commonly cause endemic disease and lower mortality. Detection therefore relies upon observation of the pathogen: detecting the presence of pathogen-specific antibodies in the blood, direct culture of the pathogen, or detecting the presence of its genetic material (DNA or RNA). However, the level of antibodies in the blood (termed serology) cannot usually distinguish between current or previous exposure to the pathogen. Non-infectious agents include both the chemical contaminants and the biotoxins produced by harmful algal blooms (reviewed in Landsberg 2002; Vos *et al.* 2003). Detecting exposure to these relies upon measuring the level of the compound (or its breakdown products, i.e. the metabolites) in the animals' tissues, and new techniques are evolving constantly to improve sensitivity, detect new toxins, and reduce the amount of tissue required for tests. Trauma is also a disease in its broadest sense, and in marine mammals, has long

been identified as an important cause of death following interaction with fisheries (Read *et al*. 2006). Although the diagnosis of some traumatic causes can be obvious, such as entanglement in a net (as the exposure), others can be harder to diagnose and rely upon detailed gross and microscopic examination of fresh tissues, e.g. gas embolism (Fernandez *et al*. 2005). Finally, other physiological, degenerative, and genetic exposures as well as causes of disease are currently not well-understood in marine mammals, and their identification requires careful and detailed sampling of individuals.

7.1.2 Confounding factors

Confounding (a term with a specific epidemiological definition, see definitions in Box 7.1 above) occurs when an independent factor is correlated with both an exposure and outcome, making it difficult to tease apart the contribution made by each to the occurrence of the disease (Rothman and Greenland 2005). A confounding factor may thus wholly or partially account for the apparent association between an exposure and a response. To cause confounding in the results however, the factors *must* be associated with both exposure *and* response. For example, primiparous females may have very high contaminant concentrations in their blubber compared to females that have already off-loaded some of their contaminants to their offspring in the milk. A study may find that the females with high contaminant concentrations have high offspring mortality, and conclude that the mortality is related to the maternal contaminant exposure. However, the true cause of increased mortality may be related to maternal inexperience rather than contaminant exposure per se. Thus reproductive status (primipary vs. multipary) is a confounding factor in this example. Potential confounding factors should be considered prior to data collection so that appropriate study design and analysis can be employed.

It is important that exposure measurements and disease diagnoses are carried out in a standardized way both within and between studies. This has certainly been addressed for some well-studied exposures such as the persistent organic pollutants, but unfortunately, for few others. Indeed, many laboratories analysing marine mammal tissues for contaminant levels are participants of the US National Institute of Standards and Technology (NIST) (Kucklick *et al*. 2002) inter-laboratory comparison and calibration scheme, and this includes some marine mammal laboratories outside the US. For example, marine mammal tissue samples collected as part of the NOAA (National Oceanic and Atmospheric Administration) Marine Mammal Health and Stranding Response (MMHSRP) biomonitoring programme for chemical analyses use the NIST protocol. Similar inter-laboratory comparison exercises were conducted in Europe for environmental samples under the QUASIMEME project (D. E. Wells and De Boer 1994). However, schemes such as these are being expanded to include infectious agents and biotoxins, but efforts to implement standardization within the marine mammal community needs to be

sustained, especially in the areas of clinical blood chemistry and haematology (Hall *et al.* 2007; Schwacke *et al.* 2009).

7.2 Effects, responses, and diagnostic techniques

7.2.1 Measuring disease occurrence

The goal of all causational epidemiological studies is to evaluate hypotheses about the causation of disease, and to relate disease occurrence to the characteristics of animals and their environment (i.e. their exposure). This requires consistent and standardized classification of disease and pathological findings as well as exposures.

Epidemiologists also use the term 'effect' in two ways. First, in the general sense, where an instance of disease may be the effect of a certain cause (for example, the effect of domoic acid causing hippocampal atrophy in California sea lions, *Zalophus californianus*, Goldstein *et al.* 2008) and second, in a very particular quantitative sense, an effect is the difference in disease occurrence between two or more groups that differ with respect to their exposure (usually termed 'exposed' and unexposed').

Various standard epidemiological measures are used to describe disease occurrence:

1 The **incidence** is the most robust measure of disease occurrence and can also be visualized as the 'flow' of disease. The incidence rate is the number of new cases (disease onsets) divided by the sum of the time over which the individual animals were observed, usually measured as 'animal time'. If 10 animals were observed for 1 year each the denominator would be 10 animal years^{-1}. If 5 were observed for 6 months and 5 for 1 year the denominator would be 7.5 animal years^{-1}. If in each case 5 developed the disease during the study period then the incidence rates would be $5/10 = 0.5$ animal years^{-1} and $5/7.5 = 0.66$ animal years^{-1}, respectively. However, measuring new disease onsets over time in marine mammals is not generally possible except perhaps in isolated cases involving long-term studies of known individuals.

2 The **risk** of a new disease occurring is quantified using the cumulative incidence (also called the incidence risk or incidence proportion). It is the proportion of individuals free from disease that *develop* a specific disease over a specified period, provided they do not die from any other disease during that period. For example, if a group of 20 animals that were initially disease-free were examined again 12 months later and 4 were found to have developed an infection, an individual's chance or risk of becoming infected over the 12-month period would be 20% (4/20).

3 The **prevalence** of disease is the proportion of a population affected at a particular point in time, and is interpreted as the probability of an individual from the same population *having* the disease at that particular point in time. Prevalence is often estimated for infectious diseases from serological data, but this is actually a

measure of an individual's encounter with infection rather than a true prevalence of disease, as the presence of antibody cannot usually distinguish between recovered and carrier animals.

The differences (i.e. the occurrence of disease in the exposed population minus that in the unexposed population) in the incidence rate, the risk (cumulative incidence), or the prevalence of disease between exposed and reference or unexposed control populations is then used to describe 'effects'. Thus the **incidence rate (IR) difference** is calculated as the incidence rate in the exposed population minus that in the unexposed and the cumulative incidence or **risk difference** is the cumulative incidence (CI) in the exposed minus the cumulative incidence in the unexposed.

Relative effect measures (ratios in the exposed compared to the unexposed) are also commonly used where the **relative excess incidence rate** is defined as:

$$\frac{\text{IR exposed} - \text{IR unexposed}}{\text{IR unexposed}} = \frac{\text{IR unexposed} - 1}{\text{IR exposed}}$$

and **the relative excess risk** (also called the risk ratio) is:

$$\frac{\text{CI exposed} - \text{CI unexposed}}{\text{CI unexposed}} = \frac{\text{CI unexposed} - 1}{\text{CI exposed}}$$

Another common relative measure is the **odds ratio**. If the CI is the probability of developing a disease over a specified period then the incidence odds is the probability of not developing the disease (i.e. 1 – CI). The odds ratio is then:

$$\frac{\text{CI exposed}/1 - \text{CI exposed}}{\text{CI unexposed}/1 - \text{CI unexposed}}$$

These various relative measures can estimate the magnitude of the association of exposures and responses, whereas absolute measures indicate the potential impact ('effect') on the population. More detail about epidemiological effect measures and their interpretation can be found in many standard epidemiological text books (e.g. Rothman and Greenland, 2005).

7.2.2 Responses, health panels, and disease classification

Diagnostic indicators

Basic haematology and blood chemistry parameters are used commonly in medicine to define health 'panels' to indicate the state of specific organ systems (Table 7.1). This approach assumes that the tests can be validated for the species of interest and that ranges of normal values for wild populations can be established. This has rarely been the case for many marine mammal species. Many studies on wild-caught or live stranded marine mammals include measuring a variety of haematological and clinical chemistry parameters (Lander *et al.* 2003; Boily *et al.* 2006) that might be

Table 7.1 *Health panels using haematology and blood chemistry parameters as indicators of the status of specific organ systems.*

Renal function	Hepatic function	Haematological status[1]	Nutritional status	Infection/ inflammation	Immune status	Skin disease	Endocrine status	Reproductive status	Cardiopulmonary status	Neurological status
Creatinine	Alanine aminotransferase	Erythrocytes	Body mass index	Leukocytes	Leukocytes	Lesion description	Thyroid hormones Thyroid-stimulating hormone response	Testosterone	Respiratory questionnaire	Seizures
Phosphorus	Sorbitol dehydrogenase	Nucleated erythrocytes	Glucose	Differential white cells	Differential white cells	Histopathologic results	Aldosterone	Oestradiol	Cytology	Behaviour (attitude, aggression)
Potassium	Aspartate aminotransferase	Haematocrit	Cholesterol	Globulins	Neutrophil phagocytosis		Cortisol	Progesterone	Oscultation	Cerebral spinal fluid aspiration, cytology, culture, serology, biochemistry
Blood urea nitrogen	Gamma-glutamyl transferase	Haemoglobin	Alkaline phosphatase	Erythrocyte sedimentation rate[2]	B- and T-cell proliferation		Adenocorticotrophic hormone response	Ultrsonography[3] of reproductive tract (uterine size, fetus detection, follicles, corpora lutea, testis size)	Culture	Computed tomography, Magnetic resonance imaging[3]

Calcium	Bilirubin (total, conjugated)	Mean platelet volume	Triglycerides	Fibrinogen	Globulins	Semen examination	Radiography, ultrasonography[3]	Electroencephalogram[3]
	Cholesterol	Red cell distribution width	Blood urea nitrogen	C-reactive protein	Interleukins		Creatinine kinase	
	Triglycerides		Albumin				Blood gases	
	Alkaline phosphatase		Electrolytes				Radiography, ultrasonography (Doppler)[3]	
	Lactate dehydrogenase		% Lipid in blubber				Lactate dehydrogenase	
	Bile acids							

[1] Excludes leukocytes; [2] ESR is not appropriate in lipaemic samples; [3] Other investigative procedures.

used for assessing health status. Some marine mammal species, such as bottlenose dolphins (*Tursiops truncatus*) and harbour seals (*Phoca vitulina*), are being extensively studied at widely geographically dispersed locations and this has led to a number of publications on reference ranges (e.g. Goldstein *et al*. 2006; Hall *et al*. 2007; Schwacke *et al*. 2009). However, once again, it is important that there is appropriate inter-laboratory calibration to eliminate the possibility of artefacts arising because of variations in analytical methods and standards. Many of the currently published 'reference ranges' should also be viewed with caution; these are often from small samples and only report the mean and standard deviations for each parameter, rather than the 95% double-sided reference intervals with 90% confidence limits on the lower and upper bounds as recommended by the International Federation of Clinical Chemistry, Expert Panel on the Theory of Reference Values (Solberg, 1983).

Other diagnostic indicators include: functional immune assays for examining both innate and adaptive immunity, physical examinations, and visual assessment of the skin, looking specifically for infectious agents (arthropods, protozoa, fungi, bacteria, or viruses) as well as neoplastic and traumatic lesions.

Disease classification

Clinical diagnoses can be categorized using disease classification schemes. One such scheme currently being implemented for marine mammals is an adapted version of the WHO International Statistical Classification of Diseases and Related Health Problems with Clinical Modifications, 10th Revision (ICD10-CM, WHO 1992). The ICD system was designed for the classification of mortality and morbidity information for statistical purposes in humans. Each major disease entity is classified in a hierarchical manner. For example, Chapter 1 of the scheme includes 'certain infectious and parasitic diseases' (codes A00–B99): A00–A09 being the intestinal infectious diseases; A07, protozoal; A07.1, giardiasis; and A07.2, cryptosporidiosis. Therefore retrieving data by code can easily identify, for example, all intestinal infections or just protozoal infections, and cases coded in this way can be retrieved for further statistical analysis. In addition, Chapter 18 of the scheme 'Symptoms, signs and abnormal clinical and laboratory findings, not elsewhere classified' (R00–R99) gives the system sufficient flexibility for the purposes of classifying the results from health assessment studies in marine mammals, using both live capture–release biochemical and health assessment data as well as post-mortem strandings' diagnoses. While it is often impossible to give a definitive cause of morbidity or death, general categories at the three-digit ICD code level can often be assigned (for example, R74 is abnormal serum enzyme levels and R74.0 is non-specific elevation of levels of transaminase and lactic acid dehydrogenase).

7.2.3 Sample and data collection

The application of epidemiological study designs requires consistency in sample collection, analysis, and reporting across populations or cohorts. The type of

epidemiological framework proposed here for marine mammals (Fig. 7.1) includes standard sample collection and analysis protocols, as well as standard assessment methods. There are three primary methods of sample collection that can be applied to marine mammal health studies, namely the recovery of stranded carcasses, field surveys, and live capture–release.

Stranding recovery

Marine mammal carcasses that wash ashore have long been a principal source of information about marine mammal pathology (Gulland 1999). In many regions formal stranding response schemes are well established, and these involve the examination of carcasses using standard procedures (Kuiken and Garcia Hartmann 1991; Dierauf and Gulland 2001) that allow comparison among different events to be made. Information can also be obtained about exposures to pathogens or toxins by sampling specific tissues that cannot be easily acquired from live animals. Although the interpretation of such data needs to take account of the cause of death, it may then be possible to, for example, estimate the total body burden of contaminants or toxins. Polybrominated diphenyl ethers can concentrate in the adrenal glands as well as the adipose tissue (Klasson Wehler *et al.* 2001), and inorganic compounds are found at higher concentrations in the liver and kidneys when compared to the blood or skin (Marcovecchio *et al.* 1990). This type of information is clearly important when estimating exposure using just a single tissue sample for monitoring.

In addition to the important disease pathogenesis, pathology, histopathology, microbiology, and other disease process information that will be gleaned during a necropsy, it is very valuable to find out why the animal died and, if possible, to determine both the primary and secondary causes of death. In reality however, this is often difficult due to decomposition but it should be the ultimate goal when fresh carcasses are examined.

Live capture–release

Very few marine mammal studies are able to live-capture large numbers of individuals at any one time. However, with recent advances in techniques and an expansion of the skills base, many pinniped and some small odontocete cetaceans are now routinely and safely captured, assessed for their health status, and then released (Harwood *et al.* 1989; Wells *et al.* 2004). The impetus for these studies spans all scientific fields and, although some are carried out specifically to determine the health or disease status of the population, a health assessment component could be carried out for many at a low additional cost. Particularly when combined with strandings and photographic or remote-sensing follow-up of the same population, these types of studies will allow inferences to be made about the health status of the population and its potential role in its population dynamics.

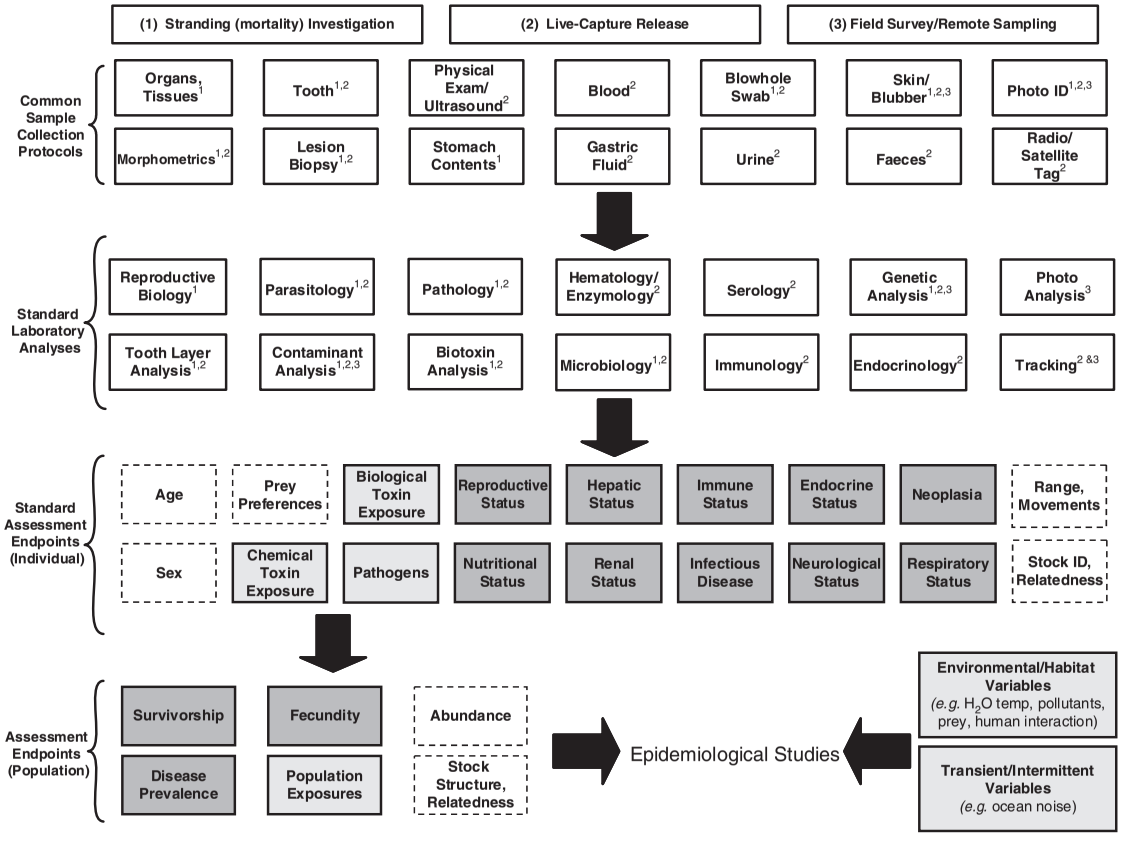

Fig. 7.1 Schematic of epidemiological framework for marine mammal studies. Types of samples are shown with superscripts indicating if they are generally collected through: (1) stranding (mortality) investigation; (2) live capture–release; and (3) field surveys and remote dart sampling. Light-grey assessment endpoint boxes indicate an exposure endpoint, darker grey indicate an effect endpoint. Dashed boxes represent important covariates for analyses.

Field survey

Visual observation, including photo-identification (see Chapter 2), has been used to a limited extent as a tool to investigate changes in endemic disease in cetaceans (B. Wilson *et al.* 2000; Pettis *et al.* 2004; Hamilton and Marx 2005). Many such photo-identification monitoring studies are conducted in conjunction with biopsy darting to collect skin and blubber samples (R. S. Wells and Scott 1990). DNA from skin samples can provide other fundamental covariate data such as sex or stock identification. In addition, the relationships between sampled animals and gene expression for particular proteins could be used in future functional genomics applications (see Section 7.2.4). Certain contaminants (e.g. persistent organochlorines, mercury) and other exposure biomarkers (such as the enzymes important in the breakdown of the contaminants, the cytochrome P450s) can also be measured from biopsy samples (Fossi *et al.* 1997). However, care must be taken when interpreting contaminant measurements in blubber samples if only the superficial blubber layers are sampled.

For some species, additional types of sample collection are also available to complement monitoring studies. For example, a visual health assessment model has been proposed for use in the endangered North Atlantic right whale (*Eubalaena glacialis*) (Pettis *et al.* 2004), with additional efforts being directed towards acquiring baseline health information from faecal samples. Faeces can provide supplementary exposure information, such as the prevalence of specific parasites (Hughes-Hanks *et al.* 2005) as well as reproductive status from hormone metabolites (Rolland *et al.* 2005).

7.2.4 Disease diagnosis

Diagnosing disease in marine mammals and necropsy sampling can be highly sophisticated. It is beyond the scope of this chapter to give details, but these subjects are meticulously covered in the books *Marine Mammal Medicine* (Dierauf and Gulland 2001) and *Marine Mammals Ashore* (Geraci and Lounsbury 2005).

Genomics

Rapid advances in genomics have created new opportunities for understanding the role of gene function in the health of marine mammals and in the diagnosis of disease. As emerging and resurging diseases become more of an issue for marine mammal conservation and disease outbreaks of unknown aetiology more common (Gulland and Hall 2007), molecular methods are likely to prove to be particularly important.

The increasing number of genome assemblies currently available may make it possible to interpolate gene function in species that are, as yet, relatively poorly studied. Functionally homologous genes in other species that are activated in response to infection or toxin exposure may be useful for studying marine mammal health. Although no complete marine mammal genome has been described, a low

coverage (×2) assembly of the bottlenose dolphin genome is available as part of the Broad Institute's Mammalian Genome Project (http://www.broad.mit.edu/mammals/). While many of the genomic tools that exist for model organisms are also not yet available for use in marine mammal species, some of the powerful, now-routine methods for screening tissues for differential transcript expression during disease states have enormous potential to identify differences in gene regulation. Some of these may already be applicable to marine mammals. However, knowing about gene expression does not necessarily lead to a functional understanding. Microarrays are enormously powerful tools that enable the activity of a vast number of genes to be determined in a target sample. DNA microarrays containing thousands of gene probes can be simultaneously exposed to a target sample. The probes, selected from cDNA fragments, are spotted onto a solid support and the expression of the corresponding messenger RNA molecules in the target sample determined. However, interpretation of this vast amount of information requires careful management and the availability of reference databases to allow proper analysis. Bioinformatic techniques have been developing simultaneously, but careful study design and statistical modelling is needed to ensure meaningful results (Kerr and Churchill 2001; Churchill 2002). The one array from a marine mammal, again the bottlenose dolphin, has been produced from lipopolysaccharide (LPS) and human interleukin-2 (IL2) stimulated peripheral blood leucocytes (Mancia *et al.* 2007). Such an array is especially useful for detecting leucocyte stimulation and specific gene upregulation. However, genes that have been downregulated in the experimental sample or genes that have been upregulated through other factors may not be represented on such an array. Dogs are the closest relatives to the pinnipeds for which an array is available. If used for marine mammals, controlling for cross-species hybridization would be critical but manageable with thorough analysis and follow-up experimentation to validate the results. However, as more microarrays from different marine mammal species become accessible this will be an extraordinarily powerful tool.

Whatever the final samples and procedures used it is essential that the collection, methods, and data reporting should be standardized. For microarrays in particular, guidelines have been established by the International Microarray Gene Expression Data (MGED) group (http://www.mged.org/Workgroups/MIAME/).

One related field that will considerably advance our understanding and identification of marine mammal disease processes is known broadly as pathogenomics (Pompe *et al.* 2005). This is the examination of both the pathogen *and* the host and how they interact with each other. The study of pathogens in humans and domestic animals is undergoing major changes, largely due to the availability of whole genome sequences, new screening technologies, proteomics, comparative genomics, and bioinformatic methods for both the host and pathogen (Lederberg 2000). Molecular fingerprinting, single-nucleotide polymorphism analyses, and molecular epidemiology all allow the study of the molecular processes

during infection in humans, and these methods could also be applied to the study of disease processes in marine mammals, particularly during rehabilitation or *in vitro*. Genetic approaches can also be used to identify novel diseases in combination with pathology and histology. Isolation and characterization of a novel papillomavirus from a bottlenose dolphin has used a pathogenomic approach (Rehtanz *et al*. 2006). This illustrates the power of isolating genetic material from a particular pathogen and using this to achieve a thorough understanding of a new pathogen.

These technological advances and their application in marine mammal microbiology will improve our understanding of host–microbe interactions and immune responses. Web-based resources such as the Virulence Factor Database (VFDB, Yang *et al*. 2008) and the Pathogen–Host Interaction Data Integration and Analysis System (PHIDIAS; Zuoshuang Xiang *et al*. 2007), coupled with the fact that complete genomic sequences of all the major pathogens from humans, plants, and animals are now available (Pallen and Wren 2007), will increase the potential for comparative genomics in marine mammals—including the determinants of virulence and other hidden aspects of disease pathogenesis.

Toxicogenomics

Toxicogenomics involves investigating the influence of a chemical compound on genes and their expression. Genomics has revealed that very few single inherited human alleles are directly associated with a specific disease risk. Over 95% of diseases are caused by a repertoire of genes that modify each other and are influenced by some kind of environmental exposure. This is a particularly pertinent area of investigation for marine mammals due to their well-documented exposure to environmental contaminants and the detrimental effects of such contaminants on their health (O'Shea 1999). There is considerable crossover in methodology and data from pathogenomics, but disease causation cannot be fully understood until the contribution of both genetics and the environment are considered.

These studies rely on a detailed understanding of the environment that the study species lives in and how and when it has been exposed to particular toxins. To some extent this can be accomplished by measuring exposure in the individuals themselves (e.g. lipid-soluble contaminants from blubber biopsy samples), but for a risk assessment approach this will often need to be combined with additional exposure information such as prey type, quality, and quantity. The ultimate aim of such research is to discover susceptibility genes and alleles to particular toxins. This will allow the testing of individuals or population screening for additional susceptibility from chemical exposure.

Web-based public databases are also available for data mining and submission such as the Comparative Toxicogenomics Database (http://ctd.mdibl.org/). This is a searchable multispecies database collating toxicogenomic data to discover relationship between chemicals, diseases, and genes.

7.3 Epidemiological study designs

The design of epidemiological studies from which robust effect measures (see Section 7.2) can be estimated is essential for determining causal links between exposures and responses. Examples of the types of study and the issues involved in each are given in Table 7.2. These approaches have been developed in the fields of human and veterinary epidemiology to test causal hypotheses (including the effect of environmental and nutritional exposures which are highly comparable to the exposure routes for marine species). Despite the extensive application of these methods in other fields and knowledge of the specific definitions of the various epidemiological terms, they have not received the kind of attention they deserve in marine mammal disease research. Many of the limitations often argued for establishing causality in marine mammal health are also problematic in human epidemiology. For example, as with humans, marine mammal health studies usually have to use exposure-response data from surrogate species.

The primary objectives of an epidemiological study are to: (i) investigate the temporal and spatial distribution of disease within the different groups, and (ii) demonstrate a causal link between one or more specific factors and the frequency of occurrence of disease. The first objective is met through the application of descriptive studies such as those involving correlation and cross-sectional studies (see Table 7.2). Studies involving correlation are aimed at identifying groups that do, or do not, develop a disease, which in turn provide clues that can lead to the formulation of causal hypotheses through the identification of differences in exposures.

In a few cases, correlation study designs have already been used to investigate associations between environmental variables and marine mammal disease. For example, B. Wilson *et al.* (1999b) conducted a correlation analysis to identify factors associated with the occurrence of epidermal disease in bottlenose dolphins. They compared the prevalence of skin disease among dolphin populations in diverse geographical areas and then attempted to correlate the disease prevalence with anthropogenic factors such as organochlorine and trace-metal contaminant exposure, as well as environmental factors such as water temperature, salinity, and UV radiation. Their analysis indicated that lesion prevalence and severity were most strongly correlated with water temperature and salinity, and were not significantly correlated with the contaminants included in the analysis.

More intensive capture–release efforts can also support such correlational analyses, but these can also be used to conduct cross-sectional studies in which exposure and health effects are assessed simultaneously in the same individuals. While these studies still suffer from an inability to distinguish whether the exposure precedes or results from the observed disease, they enable correlation between exposure and effect on an individual level, and as 'snap-shots' they provide information on the prevalence of disease and the overall health of the population for further hypothesis generation.

Table 7.2 *Epidemiological study designs.*

	Description	Issues/disadvantages
Descriptive design	Describes general characteristics of disease distribution; useful for generating hypotheses	Cannot prove causality
Case series	Detailed report on condition of single individual; can suggest the emergence of new diseases	No controls, so cannot assess differences in exposure between diseased and non-diseased individuals
Correlational	Compares exposure and effect (e.g. disease frequency) on a population basis; measures of exposure and effect are not necessarily on the same individuals within a population	Does not link exposure to effect within the same individual; 'ecologic-fallacy', i.e. correlations at the population level may not hold at the individual level
Cross-sectional	Exposure and effect (e.g. presence of disease) is assessed within the same individuals at the same point of time	Generally cannot determine if exposure preceded or resulted from the observed effect
Analytical design	Explicit comparison of exposure and disease status; can be used to test epidemiologic hypotheses	Generally requires more effort and often long follow-up periods
Case-control	A group of individuals with disease and a group of individuals without disease are chosen and their exposures are compared retrospectively	
Longitudinal	A cohort of individuals is chosen based on the absence/presence of exposure and then followed for a period of time to assess the outcome (e.g. development of disease) of interest; may also be conducted retrospectively	Often requires long periods of follow-up

Classical cohort or longitudinal studies remain among the basic analytical study designs for human and veterinary epidemiologists. Here, the investigator defines two or more cohorts (populations or groups within a single population) that are free of disease but that differ according to their exposure to the potential cause of a disease (Rothman and Greenland 2005). Usually one cohort is defined as the reference or unexposed, while one or more additional cohorts are exposed (see Section 7.2). These may represent a gradation of exposure as might be possible in, for example, the study

of the impact of contaminants on animals inhabiting a pollution gradient (Reijnders *et al.* 1999) or the extremes of exposure. Following individuals is often difficult for free-ranging marine mammals and it is often impossible to diagnose disease at the time it occurred. However, for some species it is feasible to estimate the likelihood that death or overt disease has occurred within the follow-up period. For example, weekly or monthly photo-identification follow-up of live-capture released individual bottlenose dolphins in a well-characterized population, such as the Sarasota Bay, Florida population (Wells and Scott, 1990), would enable the timing of mortality or emigration or skin lesion development to be estimated. Thus, by following the fate of known, marked individuals, it is quite feasible to estimate the relationship between disease occurrence or vital rates and exposure status. Such mark–recapture studies can be used to estimate the effect of individual or group covariates on survival probabilities (Hall *et al.* 2001), and simple modifications to these designs can be envisaged that would allow the assessment of disease occurrence instead of mortality using the same framework.

A recent study by Hall *et al.* (2006b) also demonstrated the applicability of case-control studies (in which animals are defined as cases and controls and are then stratified by their exposure status as exposed or unexposed, see Table 7.2) of marine mammal strandings data, allowing estimates of relative risks using odds ratios (see Section 7.2.1) to be determined. As with case-control studies in human medicine, careful control selection and accurate retrospective exposure assessment are required to avoid bias. However, with a sufficiently large sample size it is quite feasible that case-control studies would be equally applicable to other marine mammal strandings datasets.

7.4 Risk assessment

Once a relationship between an exposure and response has been established, the next stage is to answer the 'so what' question—in other words, what is the biological significance? How important is the relationship for the population? The key questions are how to set acceptable risks and understand how risks change under different management approaches. What is 'acceptable' is clearly outside the scope of this chapter and remains an issue that must be directly gauged for each situation and species within its context. For example, what is acceptable for a large, exponentially increasing population may be unacceptable for a small, fragmented population or an endangered species.

Risk assessment, as defined by the US Environmental Protection Agency (EPA) and widely adopted across many different organizations and in various forms, is 'the process in which information is analyzed to determine if an environmental hazard might cause harm to exposed persons and ecosystems'. And since the early 1990s this process has been formalized into a risk assessment paradigm (Fig. 7.2), largely developed within the EPA and the National Research

Council but then taken up by many different groups across a very wide range of applications.

The first step in any risk assessment is the hazard identification (or problem formulation) stage. This includes the development of a conceptual model that sets out the impetus for the risk assessment and the description of the problem, including the interactions of a particular pathogen or toxin (exposure), within a defined population and a defined exposure scenario. The conceptual model thus helps to focus the risk assessment, outline its goals, breadth, and often the policy context in which it is being conducted. The hazard identification process may be triggered by differing types of observations or events, such as: the occurrence of one (or many) unusual mortality events (Gulland and Hall 2007); information from dedicated pathogen surveys, such as those carried out by Gaydos *et al.* (2004) for southern resident killer whales (*Orcinus orca*); information on a chemical spill or pollutant concentrations measured in sediments or biota (Pulster *et al.* 2005); or the observation of a harmful algal bloom from remote sensors (Flewelling *et al.* 2005).

The characterization of exposures and responses make up the analysis phase of the disease risk assessment process, for which various factors need to be considered. These include information on the temporal and spatial distribution of exposure as well as data for disease risk assessment (see Fig. 7.2) about the survival, persistence, and amplification of the agent and its concentration in the environmental media or prey. In addition, the routes of exposure and the size, demographics, and behaviour of the exposed population must be considered. These components are brought together in an exposure profile, which ideally provides a *quantitative* evaluation of the magnitude, frequency, and patterns of exposure to a toxin or disease agent for the scenario developed during the problem formulation stage. It should also include an indication of the underlying assumptions that have been made (based on scientific judgement) and a quantification of the uncertainties associated with each element (i.e. the errors around each factor). Exposure profiles could also be estimated from modelling the movements of animals in relation to point sources of exposure. For example, Littnan *et al.* (2006) evaluated the distribution and movements of Hawaiian monk seals (*Monachus schauinslandi*) in relation to coastal waters and sources of land-based, water run-off and sewage dispersal as sources of pathogens.

The second arm of the exposure–response characterization stage (see Fig. 7.2) requires information about toxicity and about the host to enable a disease risk assessment, as this will determine the impact of the agent or organism on the individual and ultimately the population (for example the age structure, immune and nutritional status of the population, its social or behavioural traits, and its foraging behaviour).

While correlational and cross-sectional studies being carried out on marine mammals are helping to identify hazards and provide information on mechanisms of effect, analytical study designs are helping to define quantitative relationships

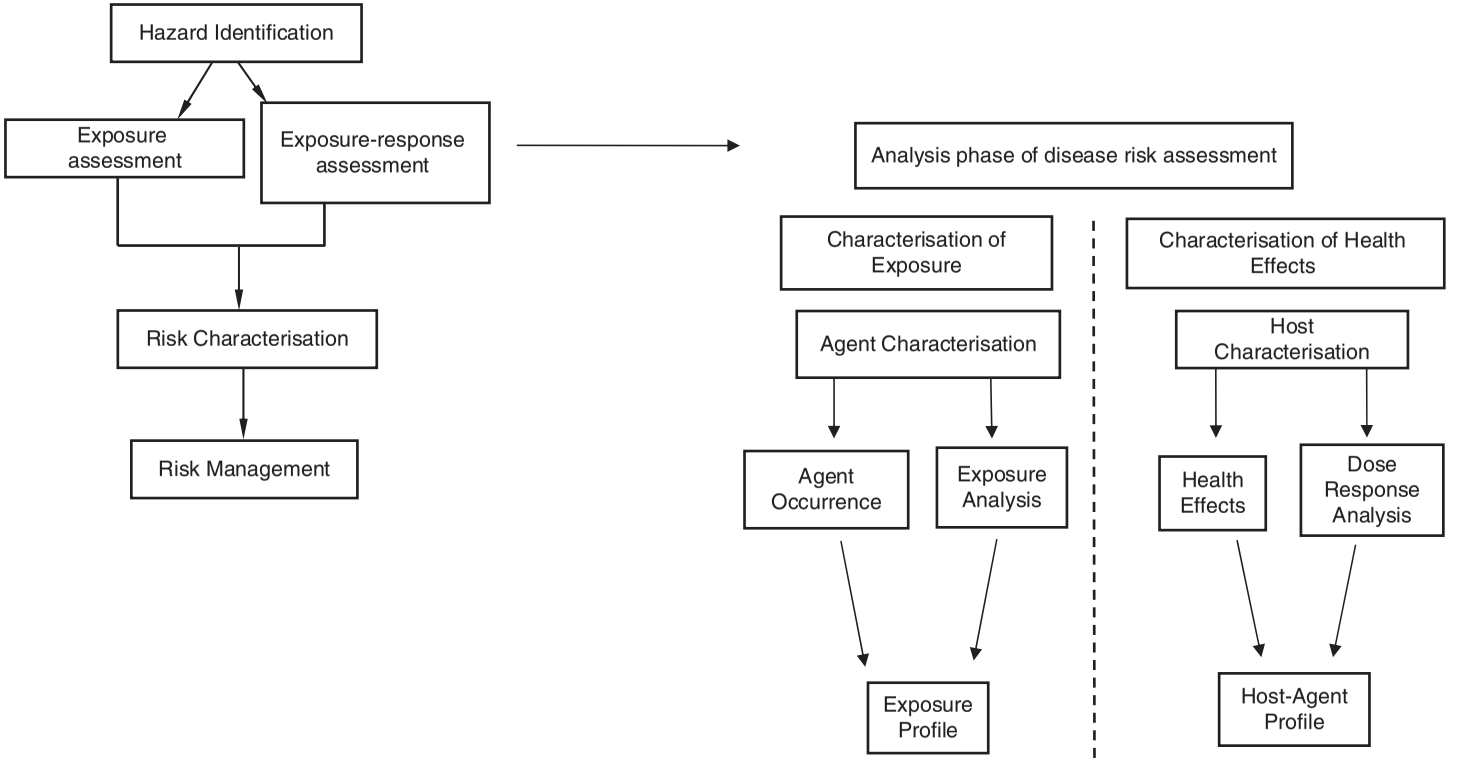

Fig. 7.2 Schematic diagram of stages in a health and disease risk-assessment procedure.

between exposure and various 'endpoints' (i.e. fecundity and survival) relevant to management, and are therefore supporting exposure–response characterization.

Dose–response relationships, as determined from laboratory studies of experimental species and widely applied for human health risk assessment, can also contribute. For example, Schwacke *et al.* (2002) took a novel approach to integrate measured tissue concentrations of polychlorinated biphenyls (PCBs) from bottlenose dolphins with a surrogate dose–response relationship (from laboratory studies in mink) to predict the health risks and associated uncertainties for dolphin populations. The results indicated a high likelihood that reproduction, primarily in primiparous females, was being severely impaired by chronic exposure to PCBs. Excess risk of reproductive failure, measured in terms of stillbirth or neonatal mortality, for primiparous females was estimated to be between 60 and 80%, whereas females of higher parity, which had previously off-loaded a large proportion of their PCB burden, had a much lower risk. These types of risk measures then become very tangible parameters that can be readily used by conservationists and managers. In another study, longitudinal data on maternal PCB tissue burdens and reproductive outcomes from the long-term Sarasota Bay dolphin study (Wells *et al.* 2005) were combined with similar data from a captive dolphin cohort (Reddy *et al.* 2001) to estimate a dose–response function linking maternal PCB burden with calf survival (Hall *et al.* 2006a). The number of individuals included for this analysis was limited and much more data are needed to increase the confidence in the dose–response model predictions in this study. But, with continued follow-up of individual dolphins as well as longitudinal study of additional populations with potentially higher PCB exposures, the necessary data are gradually becoming available.

These examples highlight one of the most difficult aspects of exposure–response assessment in marine mammal disease risk assessment. Statistical models are needed to quantify exposure–response relationships (ideally in the species of interest; but if this is not ethically or logistically possible, in surrogate laboratory model species, a situation that is the same for human risk assessments). These data are often unavailable, as many dosing studies in laboratory species do not report the final tissue concentrations that are needed for comparison with tissue concentration information collected from marine mammals. If the agent of interest is a pathogen or algal toxin, then data also required are the route of exposure, the source, and preparation of any challenge material used, as well as the organism, duration, and multiplicity of exposure.

The final phase of the risk assessment procedure then ties all the previous steps together to define population level impacts that are important for management (Fig. 7.2). The assessment then starts with a risk description of event or events, followed by a risk characterization that must include the risk magnitude and probability of potential impacts. Most importantly, the uncertainty in the risk is characterized and the confidence limits around the various estimates are reported. Sensitivity analyses can also be carried out to evaluate the most important variables

and determine the information needs and control measures. Their effect on risk magnitude and profile can then be determined. Finally, decision analyses evaluate alternative risk management strategies.

In all likelihood, the health of marine mammal populations is currently being affected by an aggregation of stressors. Identification of the most critical factors and their interactions will require relatively complex analytical approaches. A recent paper by Plowright *et al.* (2008) also highlights this issue. In a comprehensive discussion of the relationship between ecology and epidemiology they explore how causal inference may be approached by bringing together the tools and techniques that have evolved in each of these separate scientific fields. The efforts to acquire the necessary data to implement such approaches are worthwhile because they promise an opportunity for us to really understand the impacts of anthropogenic actions, both positive and negative, on marine mammal populations. Much as epidemiological studies currently guide the identification of hazards, recommendations for exposure limits, implementation of preventive measures, and allocation of resources for disease responses in human populations, they can ultimately do the same for marine mammals.