Epizootics of sudden death in tammar wallabies (Macropus eugenii) associated with an orbivirus infection

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Epizootics of sudden death in tammar wallabies (Macropus eugenii) occurred at six research facilities and zoological gardens in New South Wales, Australia, in late 1998 and at one Queensland research facility in March 1999. There were 120 confirmed tammar wallaby deaths during this period; however, population censuses indicated that up to 230 tammar wallabies may have died. The majority of animals died without premonitory signs. A small proportion of wallabies exhibited increased respiratory rate, sat with a lowered head shortly before death or were discovered in lateral recumbency, moribund and with muscle fasciculations. Gross postmortem findings consistently included massive pulmonary congestion, mottled hepatic parenchyma and subcutaneous oedema throughout the hindlimbs and inguinal region. Approximately 30% of the animals examined also had extensive haemorrhage within the fascial planes and skeletal muscle of the hindlimb adductors, inguinal region, ventral thorax, dorsal cervical region and perirenal retroperitoneal area. The tissues of affected animals became autolytic within a short period after death. Bacteriological examination of tissues from 14 animals did not provide any significant findings. Toxicological examination of the gastric and colonic contents of four animals did not reveal evidence of brodifacoume or other rodenticides. Viruses from the Eubenangee serogroup of the Orbivirus genus were isolated from the cerebral cortex of nine, and the myocardium of two, tammar wallabies and the liver and intestine of another tammar wallaby. A similar orbivirus was also isolated from the cerebrospinal fluid of another tammar wallaby that died suddenly. The disease agent appears to be a previously unrecognised orbivirus in the genus were isolated from the cerebral cortex of nine, and the myocardium of two, tammar wallabies and the liver and intestine of another tammar wallaby. A similar orbivirus was also isolated from the cerebrospinal fluid of another tammar wallaby that died suddenly. The disease agent appears to be a previously unrecognised orbivirus in the Eubenangee serogroup. This is the first report of epizootics of sudden deaths in tammar wallabies apparently associated with an orbivirus infection.

Keywords  Eubenangee serogroup; Macropus eugenii; orbivirus; tammar sudden death syndrome; tammar wallaby; wildlife

Abbreviations  ELISA, enzyme-linked immunosorbent assay; NSW, New South Wales; PRNT, plaque reduction neutralisation test; RT-PCR, reverse-transcription polymerase chain reaction; TSDS, tammar sudden death syndrome; VNT, virus neutralisation test

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for aerobic and anaerobic bacterial cultures and the remainder of the samples were stored at −70°C. Samples of brain, skin, gonads, adrenal glands, salivary glands, thyroid glands, tongue, skeletal muscle, lung, myocardium, squamous and glandular stomach, small and large intestines, liver, pancreas, mesenteric lymph node, kidney and bladder were fixed in 10% neutral-buffered formalin, processed routinely, stained with haematoxylin and eosin, and examined by light microscopy. The eyes of animals that had been dead for more than 6 h were also fixed whole in formalin and examined histologically. Whenever possible, blood within the cardiac ventricles was collected into an evacuated 7-mL glass tube containing a silicon gel separator. The serum was subsequently collected and stored at −70°C prior to serological testing.

Samples of brain, liver, lung, spleen and either kidney or intestine from 12 of the wallabies were submitted for virus isolation after several weeks’ storage at −70°C. Virus isolation was conducted by inoculating tissue homogenates onto monolayers of baby hamster kidney (BHK21) cells.4 Samples were considered to be negative for virus isolation after four passages in cell culture. Virus isolates were initially examined by transmission electron microscopy and later tested in an orbivirus serogrouping enzyme-linked immunosorbent assay (ELISA).3 Plaque reduction neutralisation tests (PRNT) were carried out using a number of viruses from the Eubenangee serogroup, including CSIRO 20, CSIRO 23, Tilligerry, and IN1074.6,7 Samples from virus-infected cells were submitted for reverse-transcription polymerase chain reaction (RT-PCR) and sequence analysis. The nucleic acids were extracted with an RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RT-PCR was then completed on the purified RNA with the One-Step RT-PCR Kit (Qiagen) and Eubeaengsee specific primers for RNA segment 3.5 Specific PCR products were identified by agarose gel electrophoresis, purified with Qiagick PCR Kits (Qiagen) and sequenced using an Applied Biosystems AB377 automated sequencer (Foster City, CA, USA). The DNA sequences were aligned using CLUSTALW 1.6 and the relationships were determined using the PHYLIP package.8,9 The phylogenetic trees were then constructed using the TreeView program.10 Gastric and colonic contents from four tammar wallabies were stored at 5°C for 5–8 days and then subjected to thin-layer chromatography for the identification of brodifacoume and other rodenticides.11 Blood was collected on 1 December 1998 from five tammar wallabies that were living in the pens where the largest number of dead tammar wallabies had been discovered. Serum samples from these animals and from the heart blood samples of four of the dead wallabies were examined for antibodies to encephalomyocarditis virus by virus neutralisation test (VNT) and Toxoplasma gondii using an ELISA. A VNT that utilised an orbivirus isolated from the tissues of an affected wallaby, together with several serotypes of Australian orbiviruses, was used to test paired sera collected before and after the outbreak. Sera were collected from 36 tammar wallabies, 3 parma wallabies (M. parma), 2 rock wallabies (Petrogale sp.), 2 red kangaroos (M. rufus) and 1 red-necked pademelon (Thylologe thetis).

Three adult tammar wallabies housed in a research facility in Yeerongpilly, Queensland, died suddenly between 22 and 30 March 1999. The first two animals were found dead and decomposed, but the third animal was found recumbent, depressed and hypothermic and died overnight. Samples of brain, lung, liver, kidney, adrenal gland, spleen, heart and skeletal muscle were preserved in formalin and processed for histological examination. Samples of several tissues and a sample of cerebrospinal fluid were collected aseptically and frozen at −70°C prior to submission for virus isolation. Several months after the initial investigation at the Sydney research facility, formalin-fixed tissues from tammar wallabies were submitted by two zoological gardens in Dubbo and Sydney, NSW, to the Australian Registry of Wildlife Health for histological examination. These animals had died during December 1998. Reports of sudden deaths of tammar wallabies in November and December 1998 were also received from two other research facilities in Sydney and Newcastle, NSW. Similar episodes of sudden deaths of tammar wallabies also occurred in two research facilities (Sydney and Newcastle, NSW) and the zoological park at Dubbo, NSW, during March and April 1998. Animal care log books from each of these facilities were examined to determine the number of tammar wallabies that died between November and December 1998.

Results

Examination of the Sydney research facility’s log book and discussions with the animal care staff revealed that the mortality rate in the tammar wallaby collection began to rise from 18 October 1998 and remained elevated until 18 December 1998 (Figure 1). Deaths followed a period of heavy rain and heavy rat infestation of the facility. Male, female, adult and juvenile tammar wallabies died during the epizootic; 85 tammar wallabies (68 adults, 17 pouch young) are known to have died at the research facility during this period, but because of the difficulties in finding dead animals among the vegetation within the enclosures and the rapid decomposition of carcasses, these data are likely to underestimate the full extent of mortality. Many pouch young died from dehydration and emaciation or were euthanased after their mothers were found dead.

Prior to the onset of the epizootic, 234 tammar wallabies were housed at the research park, which also housed approximately 450 other...
macropods of 10 species, a variety of hybrid macropods, brushtail possums (*Trichosurus vulpecula*), northern brown bandicoots (*Isoodon macrourus*) and several free-ranging mammalian species on a 6-ha site. Macropod pens in the research facility had a dirt substrate and were planted with *Pseudocapsicum* spp. At the time of the outbreak, these plants had green berries, which can contain toxic compounds. None of these plants appeared to have been eaten by the wallabies. The research facility had a large population of feral cats (*Felis sylvestris*), black rats (*Rattus rattus*) and brown rats (*Rattus norvegicus*) at the time of the outbreak. A pest control program using a rodenticide, brodifacoume, was initiated after the onset of the epizootic. The macropod enclosures were located adjacent to two large ponds used for the rearing of lungfish (*Neoceratodus forsteri*) and mosquitoes were abundant.

Although housed in similar pens and fed the same ration, deaths of any of the 10 other macropod species were not noted.

Signs of illness were not observed in most of the tammar wallabies that died during the epizootic. When clinical signs were present, they appeared to be restricted to lethargy, depression, sitting with a drooping head, ataxia, lateral recumbency with muscle fasciculations and increased respiratory rate. These animals often died during or shortly after transport to animal care facilities.

Consistent gross postmortem findings were moderate to marked pulmonary and hepatic congestion and subcutaneous oedema throughout the hindlimbs and inguinal region. Approximately 30% of the wallabies examined also had moderate to marked haemorrhage within the perirenal retroperitoneal tissues, subcutis and intermuscular fascia of the hindlimbs, inguinal, ventral thoracic and dorsal cervical regions. These haemorrhagic lesions were observed late in the course of the epizootic and were first noted in animals that died on 2 December 1998. One of the dead animals had a severe case of oral necrobacillosis in addition to massive pulmonary congestion and a mottled liver.

Marked and diffuse pulmonary and hepatic congestion and lympholysis within lymphoid germinal centres were consistent findings upon microscopic examination of the tissues collected from the tammar wallabies in Sydney, Dubbo and Yeerongpilly. Additional findings in approximately 30% of the animals examined included gastric ulceration and pericardial acute hepatocellular necrosis.

Viruses with a morphology consistent with that of orbiviruses were isolated from 9 of 11 samples of cerebral cortex of tammar wallabies from the Sydney research facility. The primary isolate (A35) was designated NSW ‘98. Viruses were also isolated from the lung of three of these animals, from the myocardium of another tammar wallaby and from the myocardium, lung and spleen of yet another tammar wallaby. A similar virus (designated Z842) was isolated from the cerebrospinal fluid of the tammar wallaby from the research facility at Yeerongpilly. Serogrouping ELISA identified the viruses as antigenically associated with the Eubenangee serogroup. PRNT serology suggested a weak cross-neutralising relationship between the primary isolate (NSW ‘98–A35) and Eubenangee isolate CSIRO 23. Molecular sequence analysis of these isolates showed they were 75% and 72% identical to the prototype Eubenangee virus isolates In1074 and CSIRO 34, respectively (Table 1). The NSW (A35) and Queensland (Z842) viruses showed a 99% homology to each other at the nucleotide level. A phylogenetic tree was constructed to define the evolutionary relationships among the orbiviruses based on gene segment 3 (Figure 2). The sequence data for these isolates can be located as Genbank Accession numbers GU078433–GU078439, inclusive.

Serum samples collected from five live tammar wallabies did not contain detectable antibodies to encephalomyocarditis virus (titre <1 : 10). Four of these tammar wallabies also lacked detectable antibodies to *T. gondii*. One wallaby had a titre of 1 : 250 for *T. gondii*. Neutralising antibodies to the orbivirus isolate were not detected in any of the paired serum samples collected from tammar wallabies and other species at the Sydney research facility.

Microbiological examination of tissues from 14 of the tammar wallabies yielded no significant organisms or mixed infection with bacteria consistent with postmortem invasion.

Thin-layer chromatography conducted using the gastric and colonic contents of four tammar wallabies did not reveal evidence of brodifacoume or other rodenticides.

Examination of the animal care log books at each facility where sudden deaths of tammar wallabies occurred in November and

Table 1. Comparative analysis of Eubenangee serogroup viruses based on the sequence of a 429-nucleotide fragment of gene segment 3 with sequence differences presented as a percentage and amino acid sequence differences in bold

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NSW, New South Wales; NT, Northern Territory.

Figure 2. Dendrogram of the phylogenetic relationships of orbivuses based on gene segment 3.
December 1998 identified a total of 120 known deaths. Census data collected in these facilities before and after the event, however, indicate that as many as 230 animals may have died during this period.

## Discussion

An initial investigation into an epizootic of sudden death of 35% of the tammar wallabies at one institution progressed to include a retrospective examination of similar epizootics at six other institutions in NSW and Queensland. Because of the scarcity of clinical signs in the affected wallabies and lack of an aetiological agent early in the investigation, the term 'tammar sudden death syndrome' (TSDS) was used to describe this epizootic.

Differential diagnoses at the time of gross postmortem examination of the tammar wallabies from the Sydney research facility included: toxicity from exposure to rodenticides, plant-based toxins, alphaphylthiouria or ionophores; encephalomyocarditis virus infection; clotridial toxemia; septicaemia; and acute toxoplasmosis. Each of these diagnoses was ruled out after thorough investigation of the animal-holding facilities, histopathological examination of tissues from 17 of the animals, microbial culture from tissues of 14 of the animals, serological testing and thin-layer chromatography of the gastric contents from four of the animals.

Because of the severity of haemorrhage and oedema throughout the pulmonary parenchyma, subcutaneous tissues and intermuscular fascia, increased vascular permeability was considered to be the most likely mechanism of disease. The list of differential diagnoses was then refined to include agents capable of causing acute vasculopathy, such as exposure to an acute toxin or virus.

The isolation of an orbivirus from one or more tissues from 11 of the 13 animals submitted for virus isolation, in conjunction with the clinical presentation and gross and microscopic postmortem findings, support a presumptive conclusion that the outbreak was associated with an orbivirus infection. Laboratory testing has characterised the agent as a virus from the Eubenangee serogroup and possibly antigenically distinct from previously described isolates. Phylogenetic analysis suggested that the TSDS-associated virus was a new genotype, which could not be aligned with previously identified genotypes. This is the first time that significant genetic variation has been observed among viruses in the Eubenangee serogroup; however, there is limited sequence information available from wildlife isolates.

Several orbiviruses have now been associated with disease in macropods. Members of the Wallal serogroup of orbiviruses have been identified as the aetiological agents responsible for epizootics of choroid blindness in kangaroos in the southern states of Australia between April 1994 and July 1996. Viruses from the Eubenangee serogroup and previously unrecognised strains of the Wallal serogroup of viruses were implicated in the sudden onset of subcutaneous oedema, pruritus and urticarial skin lesions of the lower hindlegs, tail and ears of 17 captive red kangaroos in the Northern Territory between 30 December 1998 and 16 February 1999.

Although many different animal species may be infected with orbiviruses, the clinical disease associated with infection by each viral strain tends to be host-specific. Most ruminant species may be infected with bluetongue, epizootic haemorrhagic disease or Ibaraki viruses. However, it is primarily sheep that suffer from bluetongue disease, deer that suffer from epizootic haemorrhagic disease and cattle that become ill from Ibaraki disease.

Postmortem findings associated with bluetongue, epizootic haemorrhagic disease and related orbivirus infections include oral erythema or haemorrhages, enlargement and haemorrhage of the mesenteric lymph nodes, petechiae and foci of necrosis within the skeletal muscle, abomasal ulceration, multifocal cardiac and skeletal myodegeneration, lymphoid necrosis, frothy fluid within the tracheobronchial tree, interlobular oedema and pulmonary congestion. Many of these lesions were observed in the wallabies that died from TSDS.

The clinical and pathological changes associated with epizootic haemorrhagic disease virus, African horse sickness virus and bluetongue virus are primarily the result of endothelial cell damage. Ultrastructural examination of tissues from animals infected with these viruses has demonstrated hypertrophy, the appearance of cytoplasmic projections, the alteration of intercellular junctions and degeneration of endothelial cells infected with these viruses. Alterations in the integrity of endothelial cells results in the development of microvascular thrombi and increased vascular permeability, leading to ischaemic necrosis, oedema and haemorrhage. A similar pathogenesis would account for the lesions observed in TSDS.

The outbreaks of TSDS followed periods of high rainfall and a significant increase in mosquito populations. Although many orbiviruses are spread by biting midges (Culicoides spp.), some are transmitted by mosquitoes and other arthropods. It is proposed that a mosquito-borne orbivirus from the Eubenangee serogroup is the presumptive cause of TSDS. Confirming that this orbivirus is the cause of TSDS will require transmission trials in order to fulfill Koch’s postulates. Prevention approaches implemented by several zoological parks and research institutions have included improved soil drainage around macropod holding yards, regular treatment of tammar wallabies with long-acting pyrethrin insecticides, treatment of animal shelter areas with dichlorvos-impregnated plastic strips and implementation of rodent pest control programs.

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## References


**BOOK REVIEW**


The author of *Pathology of Australian native wildlife*, Phillip Ladds, is a highly experienced Australian veterinary pathologist of international repute who has worked in academia and government. He has a particular interest in wildlife pathology and has numerous publications in this field. His aim was to bring together in one volume all the available information on the anatomical pathology of Australian native vertebrate wildlife (excluding fish). Such information is widely scattered in the literature, often in non-peer-reviewed publications, such as conference proceedings and reports, or remains unpublished. This book is aimed primarily at diagnostic pathologists, but would also be of broader interest to all veterinarians who deal with wildlife.

*Pathology of Australian native wildlife* is a hard-bound A4 book comprising 582 pages and is divided into 45 chapters. The information is organised by aetiological agent. The various wildlife species are grouped as terrestrial mammals, marine mammals, birds, reptiles and amphibians. Chapter 1 deals with the pathology of stress in each of these groups. The next 40 chapters deal with the following eight aetiological categories of disease (one wildlife group per chapter): viral; bacterial; mycotic/algal; protozoal; helminthi/annelid/pentastome; arthropod/other ectoparasitic; exogenous toxins; neoplasia and related proliferations. Chapter 42 deals with congenital, genetic and possible inherited diseases. Chapters 43 to 45 deal with nutritional and metabolic diseases, physical exertion, trauma, predation and miscellaneous injury, and diseases of uncertain or unknown aetiology.

The lesions described are macroscopic and microscopic, and are complemented by relevant information on species affected, history and clinical signs. Descriptions are clear, concise and well organised. They are illustrated by more than 400 mostly colour images of gross lesions and histologic sections. The images are well focused with good white balance and colour. Though the figures are small (5.5 x 7.5 cm), the key features are sufficiently clear at the printed magnification, and each figure is accompanied by a concise description. The information is clearly and precisely set out in a format and style that is easy to read, with in-text referencing and an extensive reference list at the end of each chapter. Tables are used to summarise certain information (e.g. nematode infections at various levels of the gastrointestinal tract) and there are eight appendices which alphabetically list the common and scientific names of Australian wildlife.

The layout, table of contents, index and use of headings and subheadings all make for easy retrieval of information and enhance the utility of the book in a day-to-day working environment. Future editions could be improved by having more keywords captured in the index, as some browsing is required to find specific information in a few chapters (e.g. chapters 42–45).

This excellent book is an important contribution to wildlife pathology, being the first of its kind to bring together information on Australian native species. The author is to be congratulated for the quality of the text and images and the breadth of material, especially considering the work involved in searching non-peer reviewed publications, and has created a foundation for future editions to incorporate new literature in wildlife pathology. This is an essential reference text for all pathologists involved with wildlife cases and for veterinarians working primarily with wildlife and exotic species. It would also be useful for any clinician who treats the occasional wildlife case.

*John Mackie*  