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A virological investigation into declining woylie populations

Running head: Virological investigation into woylie populations


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Abstract

The woylie (Bettongia penicillata ogilbyi) is a critically endangered small Australian marsupial that is in a state of accelerated population decline for reasons that are currently unknown. The aim of the present study was
to elucidate the involvement of several viral pathogens through strategic serological testing of several wild 
woylie populations. Testing for antibodies against the Wallal and Warrego serogroup of orbiviruses,
Macropod herpesvirus 1 and Encephalomyocarditis virus in woylie sera was undertaken through virus 
neutralisation tests. Moreover, testing for antibodies against the the alphaviruses Ross River virus and Barmah 
Forest virus and the flaviviruses Kunjin virus and Murray Valley encephalitis virus was undertaken through 
virus neutralisation tests and ELISA mainly because of the interest in the epidemiology of these important 
zoonoses as it was considered unlikely to be the cause of the decline. Between 15 and 86 samples were tested 
for each of the four sites in south-western Australia (Balban, Keninup, Warrup and Karakamia). Results 
indicated no exposure to any of the viral pathogens investigated, indicating that all populations are currently 
aïve and may be at risk if these pathogens were to be introduced.

Introduction

Diseases can regulate the demography of wildlife species and represent, by themselves or in association with 
other factors, a potential cause of decline (Caughley and Gunn 1996; Daszak and Cunningham 1999; Daszak et 
al. 2000; Aguirre et al. 2002). Emerging infectious diseases in wildlife are reported world wide and include a 
variety of pathogens such as viruses, parasites and protozoa (Daszak et al. 2000; Daszak et al. 2001). In 
Australia various native species are threatened by several diseases (Bunn and Woods 2005; Kirkland 2005; 
Skerratt 2005; Spratt 2005; Symonds 2005) with most of these having viral aetiology, including orbiviruses and 
herpesviruses (Kirkland 2005).
The woylie (*Bettongia penicillata ogilbyi*) is a critically endangered small Australian marsupial that has recently undergone a dramatic decline with all high density populations being affected. Naturally occurring populations had been limited to three sites in Western Australia. Of these, during the course of this study, one went locally extinct, and the other two have declined by more than 90% (Wayne *et al.* 2013a).

Detailed demographic data were available in the Upper Warren region (Fig. 1), enabling the demonstration that the decline had peculiar spatial and temporal characteristics. The decline progressed northerly at an average rate of 4 km per year and little difference was found between the yearly rates of decline over time among forest blocks in the Perup Nature Reserve (Fig. 2), which includes the eastern sites of the Upper Warren region (Wayne *et al.* 2013b). Predecline density was always high (> 1 woylie ha⁻¹) (Wayne *et al.* 2013b). The decline progressed over a period of 3-5 years in each forest block and when the density reached a low level, it appears to have stabilised (Wayne *et al.* 2013b).

Given the characteristics and demographics of the woylie decline, Wayne *et al.* (2013b) hypothesised that, while predation by introduced predators played an important role, a disease may be a concomitant, if not primary, cause of the decline. Therefore, a disease investigation was initiated as part of the woylie conservation and research project.

The determination of the presence of a pathogen in a population and its prevalence are the initial steps in a disease investigation (Artois *et al.* 2001). These allow the prioritisation of future research aimed to quantify the effects of specific pathogens at a population level. An initial qualitative assessment of the hazard posed by potential diseases was carried out to aid the woylie disease investigation (Pacioni 2010) and, for the reasons briefly outlined below, it was deemed critical to determine whether detectable immune responses to
macropodid herpesviruses, encephalomyocarditis virus (EMCV) or Orbiviruses (Wallal and Warrego serogroups) were present.

Most macropodid herpesviruses (MaHV-1 and MaHV-2 and the recently discovered MaHV-4; Johnson and Whalley 1987; Johnson and Whalley 1990; Vaz et al. 2013) belong to the subfamily Alphaherpesvirinae. Members of this subfamily generally spread rapidly and establish a latent infection in spinal or cranial nerve ganglia (Quinn et al. 2002). As with other herpesviruses, MaHV can reactivate as a result of immune suppression or other stimuli (Guliani et al. 1999). Herpesviruses are usually species-specific and highly adapted to their hosts and the hypothesis of co-evolution of these viruses with their hosts is well accepted (McGeoch et al. 1995; Mahony et al. 1999). However, the phylogenetic relationship of MaHV-1 and 2 with other viruses of the subfamily would appear to contradict the co-evolution theory (Mahony et al. 1999). Yet in spite of this, only macropod species have been found to be susceptible to infection with MaHV. For example, inoculation of MaHV-1 failed to establish a systemic infection in the common brushtail possum (Trichosurus vulpecula) (Zheng et al. 2004).

Recently, a new gammaherpesvirus, Macropodid herpesvirus 3 (MaHV-3) was described after being isolated from eastern grey kangaroos (Macropus giganteus) (Smith et al. 2008; Wilcox et al. 2011). This virus was not available for use in this study and therefore it could not be included in the serological evaluation of the woylie sera.

Macropodid herpesvirus is present in the regions where woylie populations occur and has resulted in sudden death in captive populations of grey dorcopsis wallabies (Dorcopsis muelleri luctuosa), quokkas (Setonix brachyurus), western grey kangaroos (Macropus fuliginosus) and woylies (Dickson et al. 1980; Callinan and Kefford 1981; Wilks et al. 1981). However, the widespread distribution of antibody titres in marsupials
suggests that this virus has evolved with marsupial species and may be endemic in wild populations (Webber
and Whalley 1978). High antibody levels in captive animals may reflect a higher level of virus transmission due
to crowding, increased contact rates with infected animals, or increased stress leading to expression of latent
virus (Webber and Whalley 1978).

Based on the identification of characteristic inclusion bodies, the nature of the pathology lesions and growth
of herpesvirus in cell cultures, Dickson et al. (1980) reported an outbreak of MaHV infection that resulted in
the sudden death of eight captive woylies, among other species, over a period of a week. This report indicates
that woylies are highly susceptible to MaHV infection. Clinical signs, such as ulcerations or vesicles, may be
rare and difficult to detect in field investigations and with opportunistic surveillance as conducted with wild
woylie populations, it could be difficult to detect the presence of MaHV disease in a population. In this
scenario, an affected population could undergo a steep decline, with increased mortality after introduction of
the virus, similar to what the available monitoring data suggest.

The encephalomyocarditis virus (EMCV) is shed in the faeces and urine of rodents that are the natural hosts.
The infection develops in a few days and the virus can replicate in a broad spectrum of hosts including
primates, pigs, rodents and marsupials (Reddacliff et al. 1997; McLelland et al. 2001; Quinn et al. 2002).
Encephalomyocarditis virus is a known pathogen in rural areas in Australia and it can occur when rodents
build up to plague proportions around piggeries. The disease in pigs is acute with sudden death or acute
neurological signs. EMCV was isolated from a variety of Australian native fauna, including macropods
(Macropus rufogriseus, M. rufus and Dendrolagus goodfellowi), causing sudden death as the only sign
(Reddacliff et al. 1997). More recently, a case of sudden death caused by EMCV was reported in a Lumholtz’s
Tree Kangaroo (Dendrolagus lumholtzi) (David Blyde, pers. comm.), as well as an infection in a quokka
(McLelland et al. 2001).
Viruses in the genus *Orbivirus* are generally transmitted by arthropods, particularly *Culicoides* species. Infections, especially by the Wallal and Warrego serogroups, have been reported in several species of macropods that are closely related to woylies, with serious clinical consequences (Daszak *et al.* 2000). Therefore, woylies may be potentially susceptible to these viral pathogens. At its extreme, a severe clinical form of orbivirus infection, similar to that described for other kangaroos (e.g. western grey kangaroos), could have critical consequences in woylie populations. A number of orbivirus epidemics were reported in wild populations of macropods in Australia between 1969 and 1996, including outbreaks in the South West region of Western Australia (Hooper 1999; Hooper *et al.* 1999). The virus was isolated from eye and brain tissues collected from diseased kangaroos near Albany, Esperance and Perth (Hooper *et al.* 1999). Hooper *et al.* (1999) also reported a high seroprevalence of antibodies for these viruses in wild kangaroos and wallabies throughout the region. Consequently, it can be concluded that these viruses are well established in the region.

Lastly, we also tested woylie sera for the presence of antibodies against flaviviruses and alphaviruses (Ross River virus, RRV, and Barmah Forest virus, BFV). The viruses RRV and BFV are regularly isolated from mosquitoes collected in the south-west of Western Australia by the Arbovirus Surveillance and Research Laboratory at the University of Western Australia, and the seroprevalence in marsupials can be high (Lindsay 1995). The flaviviruses Edge Hill virus, Kokobera virus and Stratford virus have also been isolated from mosquitoes in the south-west of Western Australia, however little is known about the prevalence of antibodies against these pathogens in native fauna in the southwest of Western Australia (Jasinska *et al.* 1997; Johansen *et al.* 2005a). Macropods are known to have been infected with specific flaviviruses and alphaviruses but have not shown any evidence of disease, and sero-epidemiological studies suggest they could be possible virus reservoirs (Aldred *et al.* 1990; Harley *et al.* 2001; Russell 2002; Johansen *et al.* 2005b).
Knowing whether this species of macropod is contributing to maintain the virus in the habitat would also be of great interest in terms of human health as some of the infections caused by these viruses are important zoonoses. Furthermore, a positive serological result could give an indication of the exposure of this species to biological vectors such as mosquitoes. For these reasons we also included this group of viruses in the serological screening conducted in this study.

Materials and Methods

Woylies were trapped using a standard live cage-trapping technique in the Upper Warren region and Karakamia sanctuary (Fig. 1) between March 2006 and November 2008. Blood samples were collected from the lateral tail vein via a 23- or 25-gauge needle in a 2.5 or 3 ml syringe and then transferred into plain tubes. After blood clots had formed, samples were chilled in an insulated foam container with ice in the field. Upon return from the field, blood samples were centrifuged as soon as possible after formation of blood clots (but never later than 8 h after collection), and sera separated and frozen at -20°C until tested.

The selection of samples to be tested for specific tests required careful determination given the small amount of serum obtained from sampled animals and the limited number of samples available, especially from low density forest blocks. Under the hypothesis that the tested virus was the cause of the decline, two main criteria were used to select the samples: (1). The pathogen should be present in all forest blocks because it was theorised that declines within each block were not independent (Wayne et al. 2013b); and (2) Seropositive individuals were more likely to be detected in the population after the decline because survivors would be likely to have immunity (Thompson et al. 1992; Roelke-Parker et al. 1996; Tompkins et al. 2002; Härkönen et al. 2006; Thrusfield 2007).
Consequently, only postdecline samples from Balban forest block (Fig. 2) were tested for EMCV and the two Orbivirus serogroups. Woylies from a broader geographical area were tested for MaHV including a control population (Karakamia) and 15 samples were predecline (Keninup forest block). The population at Karakamia was considered a control population because this is a high density population that has not declined. It would be expected, therefore, that this population would not show evidence of exposure to the virus. When available, multiple samples collected at different times from the same individual were also tested (Table 1). The maximum possible prevalence (i.e. power analysis) with 90 and 95% confidence was calculated following the methods of Cannon and Roe (1982), using Episcope 2.0 (Thrusfield et al. 2001) with an estimated population size of 1,000 (Groom 2010).

All sera were heat inactivated at 56°C for 30 min before to testing.

For MaHV, virus neutralisation tests (VNTs) were carried out as follows: 10 µL of sera were diluted in 20 µL of phosphate buffer solution (PBS) and mixed with 10 µL MaHV-1 control virus containing 100 TCID$_{50}$ of the respective MaHV-1 and incubated at 37°C for one hour. The virus/serum mixture was then added, in 24 well plates (Nunc, Roskilde, Denmark), to the monolayer of Potorous tridactylus kidney cells previously washed from the media with sterile PBS. After the addition of 1 mL of maintenance media, the cell culture plates were incubated at 37°C in an atmosphere containing 5% CO$_2$. A ‘cells-only’ well (i.e. a well with no virus and no serum) and virus control was included in each plate. Plates were observed daily for 7 days for presence of cytopathic effect (CPE) in the monolayer. It should be noted that antibodies against MaHV-2 and MaHV-4 would also neutralise also MaHV-1, although with different sensitivity (Vaz et al. 2013).
The VNTs for EMCV and the two *Orbivirus* serogroups were carried out in 96 well tissue culture plates (Cooke Engineering Co., Alexandria, VA). Dilution series (from 1:4 to 1:32) of sample sera were mixed with a constant virus concentration of 100 TCID$_{50}$/25 μL and baby hamster kidney (EMCV) or BSR (*Orbivirus*) cells suspended in Dulbecco’s Modified eagle Medium (DMEM; EMCV) or a 1:1 mix of DMEM and Basal Medium Eagle (*Orbivirus*), and incubated at 37°C in an atmosphere containing 5% CO$_2$. A control plate was used for each test. This included a positive and negative control, a dilution series of the positive control, cell control wells with no added virus or serum, a virus titration, and a working strength titration. Additionally, sample sera were diluted 1:4 with media and an aliquot was added to a well with no virus to check for toxic effects on the cell cultures. Within 24-48 hours of incubation, cell cultures were checked for proper attachment to the bottom of the well and at the fifth day for CPE.

Woylie sera were screened for the presence of antibodies to RRV and BFV by VNT as described by Johansen et al. (2005b) with the exception that Vero cells were used instead of BHK cells. Briefly, serially diluted sera were incubated with 50-100 TCID$_{50}$s of virus diluted in M199 (supplemented with HEPES, antibiotics and L-glutamine) containing 2% fetal bovine serum (FBS) at 37°C in a 5% CO$_2$ atmosphere. After one hour approximately 1.6 x 10$^4$ Vero cells in M199 containing 10% FBS were added to each well of the 96 well tissue culture plate and the plates were incubated for five days at 37°C in a 5% CO$_2$ atmosphere. Each well was examined microscopically for CPE and neutralisation titres were expressed as the reciprocal of the highest serum dilution where CPE did not occur. Samples with neutralisation titres of 40 or more were considered positive. Virus control assays were performed each time the neutralisation assay was conducted and the assay was repeated if the infectious titre of virus used was below or above 50-100 TCID$_{50}$s. Each test serum was also added to wells containing Vero cells without virus as serum controls, and kangaroo and rabbit sera containing antibodies to RRV and BFV, respectively, were used as positive control sera.
The presence of flavivirus antibodies in woylie sera were investigated using the flavivirus specific mouse monoclonal antibody 3H6 in a flavivirus epitope blocking ELISA (Hall et al. 1995). Optimal concentrations of cell lysate antigen, 3H6 and horse-radish peroxidise (HRPO)-conjugated goat anti-mouse antibody was initially determined using a checkerboard assay. Cell lysate antigen (MVEV) in 0.05 M carbonate/bicarbonate coating buffer pH 9.6 was added to U-bottom flexible plates overnight at 4°C, leaving two wells containing buffer alone as background inhibition control wells. After removing excess antigen and washing twice with PBS containing 0.05% Tween 20 (ELISA wash buffer), 50 µl of woylie test sera and positive and negative control chicken sera diluted 1/10 in ELISA blocking buffer (0.05 M Tris, 1 mM EDTA, 0.15 M NaCl, 0.05% Tween 20, 0.2% high nitrogen casein, pH 8.0) were added to duplicate wells on each plate. Plates were incubated for two hours at room temperature, before 50µl of 3H6 diluted in ELISA blocking buffer was added to each well. Plates were incubated for one hour at room temperature before plates were washed four times to remove excess serum and antibody. Fifty microlitres of HRPO-conjugated goat anti-mouse antibody diluted in ELISA blocking buffer was added to each well for one hour at room temperature before being washed six times. Enzyme activity was visualised by the addition of 100 µl of 2,2-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) substrate buffer (Hall et al. 1995) for one hour at room temperature and results were obtained quantitatively by measuring the optical density at a dual wavelength of 415 and 490 nm using a BioRad ELISA plate reader. The percentage inhibition of 3H6 was calculated for each serum. Samples with a percentage inhibition of 30% or greater were considered positive.

**Results**

No samples neutralised MaHV-1. Complete degradation of the monolayer was apparent within 5-7 days. No CPE was observed in any cells-only wells.
Similarly, no virus neutralisation was observed in any of the sera samples tested for the presence of antibodies to EMCV or Warrego and Wallal serogroups. Initially two samples tested positive for antibodies to the Wallal serogroup virus to serum ratios of 1:4 and 1:8. However, the virus titre in the virus control was below the target titre so the test was considered invalid. When the test was repeated the samples were both positive at a serum dilution of 1:4, however the challenge virus titre was still just below the target titre. Unfortunately, the lack of sera prevented further repeats of the Wallal serogroup test. At this titre these samples were considered to be negative (Reddacliff et al. 1999).

No samples were positive for antibodies to flaviviruses in the epitope blocking ELISA. Similarly, no virus-specific antibodies were detected in the RRV and BFV VNT assays.

**Discussion**

Overall, there was no serological evidence of any of the tested viruses affecting the populations. However, due to the limited sample size, it was not possible to exclude infection from these viruses but only to exclude a seroprevalence above the maximum possible prevalence (Table 1).

There is direct evidence that woylies are susceptible to MaHV, as infection in a captive colony of woylies housed at the Perth Zoo resulted in a case fatality rate of 100% in one week (Dickson et al. 1980). However, there is no information available about possible morbidity and fatality rates caused by MaHV, or the other viruses tested in this study, in wild woylie populations to judge whether disease caused by one of these viruses may be responsible for the declines and result in post-decline prevalences lower than the maximum
possible prevalence (3.4-7.5%, Table 1). Additionally, it could be expected that, after an epidemic with a very high fatality rate, as it would be expected with MaHV, only a very small fraction, if any, of the population would have detectable antibody levels, despite the significant regulation that the disease would impose on the population demography. In such a scenario, where susceptible animals were exposed to a highly fatal disease, all, or almost all, infected animals would die and therefore it would not be expected that the disease could be detected (serologically) unless sick animals were trapped prior to death. A similar situation was modelled in fox (Vulpes vulpes) populations where, assuming that a viral respiratory infection causes the death of 50% of infected animals, a prevalence of 0.18% would be sufficient to regulate the demography of the populations (Anderson 1995).

The results of this virological investigation contributed to the baseline data needed to improve the understanding of the population dynamics of woylies and to better assess risks for management of, and translocation among these populations, as well as the establishment of new populations. Regardless of whether these viruses are present in the populations at a lower prevalence than the maximum detectable level from this study, or whether they are completely absent, a substantial proportion of individuals has not been exposed to these viruses and therefore these populations may be particularly susceptible. Anthropogenic introduction of these pathogens in woylie populations must be avoided and preventive measures adopted. While current hygiene protocols adopted by Department of Parks and Wildlife (formerly Environment and Conservation) are extensive (Chapman et al. 2011) and transmission of these viruses is unlikely through movements between sites by people and equipment (as long as the protocols are followed meticulously), management protocols to prevent the spread of these diseases when dealing with live animals are established on a case by case basis (Chapman et al. 2011). These procedures should include quarantine and routine health screening, and if unhealthy animals are encountered, they should be retained at a
quarantine facility for clinical examination to determine the causes. These recommendations have been
standard practice as part of monitoring and research activities in the Upper Warren since 2005 (DEC 2008).

Given the risks, quarantine and viral serological screening is recommended before wild rehabilitated woylies
or individuals sourced through a captive breeding program are released back into the wild, if these woylies
were housed in direct contact or in proximity to other macropods. Clinical, latent and reactivation infections
with MaHV have been widely demonstrated in other species of macropods, especially in captive populations
(Webber and Whalley 1978; Finnie 1980; Kerr et al. 1981; Guliani et al. 1999), and it could have devastating
consequences if introduced into woylie populations. When infection with EMCV is potentially possible (for
example, captive groups with known presence of rodents in enclosures), the establishment of quarantine
periods (associated with good rodent control in the quarantine facility) before releasing woylies into the wild
is recommended. This preventive measure can be considered sufficient to stop the introduction of this
pathogen into the target population, since, except in rodents, infection with EMCV generally causes sudden
death shortly after infection (Reddacliff et al. 1997; McLelland et al. 2001; Fowler and Miller 2003; Jackson
2003). It is more difficult to put in place a control protocol for Orbivirus infections, since these viruses are
transmitted by arthropods, making the exposure to these viruses a possible eventuality, especially in areas
were Wallal and Warrego viruses are known to occur (e.g. south west Western Australia, Hooper 1999;
Hooper et al. 1999). However, infection with Wallal and Warrego viruses results in clinical signs in macropods
(Fowler and Miller 2003; Jackson 2003), and it would be expected that these signs would be evident during
the quarantine period.

Every reasonable effort should also be made to prevent the possibility of wild animals that may be positive for
any of the investigated viruses (EMCV, Wallal or Warrego serogroups or MaHV) being translocated to
different sites, either elsewhere in the wild or to captive populations. While it may not be practical to screen
and test candidate animals for movement beforehand, prior screening of the source population can provide an indication of the likelihood and risks of individuals within the population being infected—as demonstrated by this study for the woylie populations in the Upper Warren region and Karakamia.

The absence of flavivirus antibodies in woylies is perhaps not surprising as isolations of flaviviruses (including Edge Hill virus, Kokobera virus and the closely related Stratford virus) from mosquitoes collected in the southwest of Western Australia is relatively rare and of unknown epidemiological significance. Serosurveys for flavivirus antibodies in marsupials in this region have not previously been conducted. The only BFV serosurvey in the southwest of Western Australia also suggested BFV infection was rare in the few small native animals tested (Johansen et al. 2005b). However, high prevalence of BFV antibodies in kangaroos were detected after an outbreak of BFV in 2000/01 and 2001/02. A previous serological survey of antibodies to RRV showed that this virus was common in western grey kangaroos, however few other marsupials were tested. A single western quoll (or chuditch, Dasyurus geoffroii) and four quokkas had antibodies to RRV (Lindsay 1995), suggesting that these species may occasionally become infected with this virus, and that the mosquito vectors of RRV occasionally take blood meals from smaller native animals.

In conclusion, this study provides important baseline information that will aid future population health screenings and inform the necessary precautions that need to be taken during the management of existing populations, future translocations or the establishment of captive populations. The study has also improved our understanding of the likelihood of whether these viruses may have been involved in the recent woylie declines. Further studies will be required to confirm complete naivety of woylie populations to the viruses tested but the data presented here are an important foundation on which to build.
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**Figure legends**

**Fig. 1.** Location of large woylie populations in Western Australia. Natural occurring populations: Kingston, Perup, Tutanning and Dryandra. Translocated populations: Karakamia and Batalling.

**Fig. 2.** Location of the two distinct woylie populations (Pacioni et al. 2011) (upper-case letters) and monitored forest blocks (lower case letters) in the Upper Warren region.
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EMCV: Encephalomyocarditis virus. WAR: Warrego serogroup. WAL: Wallal serogroups. MaHV: Macropod herpesvirus. RRV; Ross River Virus. BFV: Barmah Forrest Virus. Pr 90%-95%Conf: maximum possible prevalence at 90% and 95% confidence, based on total number of individuals tested and assuming a population size of 1000 animals.

Sample sizes reflect availability of sera (i.e. the quantity of sera available was not sufficient to carry out tests for all viruses in all locations).
Fig. 1. Location of large woylie populations in Western Australia. Natural occurring populations: Kingston, Perup, Tutanning and Dryandra. Translocated populations: Karakamia and Batalling.
Fig. 2. Location of the two distinct woylie populations (Pacioni et al. 2011) (upper-case letters) and monitored forest blocks (lower case letters) in the Upper Warren region.