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Evidence for insect transmission of rabbit haemorrhagic disease virus

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SUMMARY

The spread of rabbit haemorrhagic disease (RHD) virus from quarantine on Wardang Island to mainland Australia in 1995 suggested that insects could be potential vectors. Field observations and laboratory experiments were conducted to address aspects of this hypothesis. Firstly, the variation in insect populations on the island during the field trials was examined. There was approximately a 1000-fold increase in the number of bushflies, *Musca vetustissima*, shortly before the spread of the virus. Secondly, *M. vetustissima* were tested in the laboratory as potential vectors of RHD virus, and it was demonstrated that disease could be transmitted between rabbits by flies. Finally, 13 of 16 insect samples, collected from Wardang Island and from several sites on the mainland following the spread of virus off the island, were positive for the presence of RHD virus by a specific polymerase chain reaction (PCR). Only one sample contained sufficient infectious virus to kill a susceptible rabbit. These data, combined with previously published information on fly biology, suggested that flies, particularly bushflies, may be involved in the transmission of RHD virus. Other possible routes of spread were not assessed in this study.

INTRODUCTION

Rabbit haemorrhagic disease (RHD), which is specific to the European rabbit, *Oryctolagus cuniculus* (L.), is caused by RHD virus, a member of the Family Caliciviridae. It was first described in domestic rabbits in China in 1984 [1], and rapidly spread to Europe where it was also associated with high morbidity and mortality in domestic rabbits. It was only when the disease was observed in wild rabbits in Spain in 1988 that its potential for controlling wild rabbits in Australia and New Zealand was recognized. In both of these countries, rabbits cause significant losses to agriculture and severely damage natural ecosystems [2].

Preliminary work on RHD virus in Australia to assess its potential for use in biological control of wild rabbits was conducted at the Australian Animal Health Laboratory (AAHL), a high-security laboratory designed for the containment of exotic infectious agents. This work involved the development of diagnostic procedures to work with the virus [3, 4], susceptibility testing of, and transmission trials in, Australian and New Zealand wild rabbits in the laboratory [5], and species-specificity testing of RHD virus [5, 6]. Field experiments, under quarantine conditions, were then conducted on Wardang Island, an Australian off-shore island, to evaluate the virulence,

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transmissibility and persistence of the virus, and to consider animal welfare issues associated with its use (Cooke et al., unpublished observations).

During the course of this field work, RHD virus spread, firstly to outside the quarantine compound on the island, and, shortly after, from the island to the Australian mainland. The disease was first identified on a mainland peninsula adjacent to the island, a distance of approximately 4 km. However, about 2 weeks later, further well-established foci of the disease were discovered in a sparsely settled area of inland South Australia, approximately 270 km north-east of the island. The location of Wardang Island, and the subsequent spread of RHD throughout relatively remote areas of Australia has been well documented [7].

These events raised major questions about transmission of RHD virus. Based on the hepatic lesions caused by the virus, Morisse and colleagues [8] hypothesized that the faecal–oral route was the main form of transmission. The work of Lenghaus and colleagues [5], in which they demonstrated easy transfer of the disease between rabbits living in artificial warrens under insect-free laboratory conditions, suggested that close contact alone was sufficient for transmission.

Indirect transmission by insects also had to be considered as a means of spreading RHD virus. Laboratory work had indicated that blowflies (*Phormia* sp.) could transmit the virus [9], and Lenghaus and colleagues [5] had shown that, under highly controlled conditions, the mosquito, *Culex annulirostris*, and fleas, *Spilopsyllus cuniculi* and *Xenopsylla cunicularis*, could also transmit the disease in the laboratory.

For these reasons, seasonal changes in insect numbers on Wardang Island were monitored during the field experiments. Samples of insects were also collected from the island for the attempted detection of RHD virus. Later, after the spread of virus off the island, detection of virus was also attempted in insects collected from several sites in South Australia, Victoria and western New South Wales. Finally, soon after the spread of virus to the mainland, laboratory experiments designed to test the potential of bushflies, *Musca vetustissima*, as vectors of the disease were conducted at the AAHL. The aim of this paper is to present the data from these studies, and, in combination with previous knowledge of fly biology, assess the role of flies in the spread of RHD virus from Wardang Island. Other possible routes of spread were, however, not assessed in this study.

MATERIALS AND METHODS

Insect trapping data from Wardang Island

Experimental trials with RHD in wild rabbits were conducted in the quarantine compound on Wardang Island, off the coast of South Australia. The island lies 9 km west of Port Victoria (34° 31′ S, 137° 21′ E), and 4 km south-west of Point Pearce. It has a Mediterranean-like climate, with a mean temperature in winter of 12 °C and in summer of 24 °C. Mean annual rainfall is approximately 375 mm. The quarantine compound occupied an area of about 0.5 km², and was surrounded by two fences 20 m apart to exclude rabbits and cats. Access to the island was by boat or plane, and, in general, this was restricted to project staff.

Apart from geographical and physical barriers to the spread of RHD virus, numerous other procedures were also established in an attempt to maintain microbiological security. These included: (1) a rabbit-free zone, 300 m wide, around the quarantine compound; (2) weekly monitoring of free-living rabbits in warrens outside the quarantine compound; (3) multiple changes of clothing and footwear by staff entering, and leaving, experimental sites containing infected rabbits; (4) restrictions on staff who visited the quarantine compound from working with rabbits outside the compound for 7 days; and, (5) insect control measures, including observation of insect numbers on the island.

Insect populations were monitored monthly using five wind-orienting fly traps on the perimeter of the quarantine compound throughout the field trials. Flies captured in freshly baited, wind-orienting traps during 4 days of each month were sorted, counted and stored at −20 or 4 °C. Mosquito populations were monitored with 8 CO_2/light traps on 17 nights of the field trials. Those captured were also sorted, counted and stored.

Laboratory transmission trials with bushflies

Bushflies (*Musca vetustissima*) were supplied by K. Wardaugh, CSIRO, Division of Entomology, Canberra, Australia. Adult flies were held in small cages, and were supplied water and sugar *ad lib*, except prior to each transmission experiment when at least 60 flies were deprived of food and water for 8–19 h.

Because of a shortage of susceptible domestic rabbits, the rabbits used for testing were wild-caught. They were captured from rural areas around Bendigo

in Victoria, and Canberra in the Australian Capital Territory. In the laboratory, they were held individually in standard laboratory rabbit cages to which they adapted very quickly. Rabbits were tested for antibodies to RHD virus [3], and only seronegative animals were used.

All experiments were conducted within the microbiologically secure area at the AAHL. Three rabbits were held in an animal room, and, of these, two (nos. 871 and 876) were inoculated intramuscularly with 10^4 median rabbit lethal doses (RLD_{50}) of a Czechoslovakian strain (CAPM V-351) of RHD virus. The third was held as an uninoculated control. At 8 h post-inoculation (p.i.), each rabbit was placed in a separate, ventilated, insect-proof plastic box (0·1 m^3), and exposed to different batches of 20 food- and water-deprived flies for approximately 50 min. All of the flies with each rabbit were then collected with a vacuum device into separate plastic bags, and immediately transported to another secure room where susceptible wild rabbits were housed individually. The 3 batches of flies, 2 exposed to the inoculated rabbits and 1 to the control rabbit, were then each exposed to a separate susceptible rabbit, each in a plastic box identical to those already used, for approximately 50 min. The flies from each box were then recaptured again, with another vacuum device, and stored at −20 °C as individual batches. This procedure was repeated with the inoculated and control rabbits at 29 h p.i. when one inoculated rabbit had died. At 52 h p.i., when both inoculated rabbits were dead, their carcasses were opened with a ventral midline incision from the xiphoid to the inguinal region to allow flies access to the abdominal viscera. The uninoculated control rabbit was killed humanely, and its abdominal cavity was also opened, exactly as for the inoculated rabbits. The procedure was slightly modified at 76 and 124 h p.i.; rather than allow flies 50 min exposure to the inoculated and control carcasses, they were recaptured immediately after they began feeding around the mouth, eyes or nares, or on the abdominal viscera of the carcasses. As previously, they were then transferred to the susceptible rabbits. Fly and rabbit behaviour were observed closely whenever the two species were confined in plastic boxes. Susceptible rabbits that had been exposed to flies were held in individual cages, and observed daily either until they died or until the experiment was terminated at 19 days p.i. The liver of all dead rabbits was examined for RHD virus antigen with a specific antigen-capture ELISA [4].

With the exception of a few modifications, polymerase chain reaction (PCR) analyses of fly samples were conducted as described previously [6]. All frozen fly samples were thawed and ground in 2 ml of phosphate-buffered saline (PBS) in a mortar and pestle. After centrifugation of the suspension, 200 μl of filtered supernatant fluid was mixed with 20 μl of 10% sodium dodecyl sulphate, and then extracted, firstly, with an equal volume of phenol, and then with ether. Nucleic acids were precipitated from the aqueous phase, centrifuged at 13 000 ***g*** for 10–15 min, and, after vacuum drying, were resuspended in 5–10 μl of water. Complementary DNA was prepared using primer P1, and the PCR performed with primers P1 and P2. A specific PCR product, of approximately 258 bp, was detected by gel electrophoresis.

Insect samples from RHD epizootics

During the spread of RHD in the Australian mainland states of Victoria, South Australia and New South Wales, mixed populations of flies and mosquitoes were trapped at a number of sites, again using wind-orienting fly traps and CO_2/light traps. Samples of the species collected were sent to the AAHL where they were tested. Sixteen samples were selected from six different regions.

Insects in each sample were suspended in PBS at a minimum ratio of 5:1 (volume:weight), and then ground in a mortar and pestle. The number of insects in each sample varied considerably; while up to 50 flies were present in some samples, there were always far fewer mosquitoes. After gentle centrifugation (750 ***g*** for 10 min), the supernatant fluid from each sample was collected, and frozen at −20 °C. Each of these samples was then thawed, and filtered through a 0·2 μm filter (Minisart, Sartorius AG, Germany), and 0·5 ml of the filtrate was retained for examination by the RHD virus-specific PCR (described earlier). From 10–100% of the remaining filtrate from each sample was then inoculated intramuscularly into susceptible rabbits. Rabbits were held individually in cages, and were monitored daily. Liver and spleen from dead rabbits were examined for virus with an antigen-capture ELISA [4].

RESULTS

Insect trapping data from Wardang Island

A summary of the flies trapped in the wind-orienting traps around the perimeter of the quarantine

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Table 1. *Total flies trapped in wind-orienting traps from around the perimeter of the quarantine compound on Wardang Island during field trials with RHD virus* (*June–October, 1995*)

Species trapped	Number trapped				
	28 June* (14)†	21 July (23)	19 Aug. (29)	24 Sept. (36)	28 Oct. (29)
Musca vetustissima	1	4	0	2273	1340
Calliphora dubia	120	188	202	888	467
Calliphora stygia	22	289	602	4000	519
Chrysomya rufifacies	4	0	0	0	18
Lucilia cuprina	1	0	0	67	77
Total	148	481	804	7228	2421

* Date that traps were cleared.
† Number of trapping days.

compound on Wardang Island during the course of the field trials is presented in Table 1. The most common species trapped included: *Musca vetustissima* (Walker), *Calliphora dubia*, *C. stygia* (Fabricius), *Chrysomya rufifacies* (Macquart) and *Lucilia cuprina* (Wiedeman). Other flies [e.g. *C. robusta, Ophyra rostrata* (Robineau-Desvoidy), and Sarcophagidae] were also present. However, these were less common, and were not included in Table 1.

There was clearly a huge increase in the number of trapped *M. vetustissima* during late winter-spring (August, September and October, 1995), even allowing for the irregular interval between the emptying of traps. In general, there were also increased numbers of four other species of fly trapped over the same period. However, while there was approximately a 1000-fold increase in trapped *M. vetustissima*, the increase for the other species was closer to 10-fold.

Attempts to trap mosquitoes on Wardang Island were limited to 17 nights between 31 May and 19 October 1995. The results of that work are shown in Table 2. In general, only small numbers of mosquitoes (a mean of 3·3 per night) were caught in CO_2/light traps. Two of the 17 trapping nights were windless; 22 mosquitoes were trapped on 1 of these nights in July, and 13 were trapped on the other in September.

Laboratory transmission trials with bushflies

Of the 2 rabbits that were inoculated with 10^4 RLD_{50} of RHD virus to start the trials, 1 was dead by 29 h p.i., while the other was alive, but moribund, at 52 h p.i., when it was killed humanely. Both inoculated rabbits, but not the uninoculated control, had gross lesions consistent with RHD. With the RHD virus antigen-capture ELISA, viral antigen was detected in the liver from the carcass of each inoculated rabbit but not from the control.

There were no obvious effects on the experimental flies from the short-term, pre-trial deprivation of food and water except that, when finally exposed to infected and control rabbits, these flies were very aggressive in their attempts to land on the rabbits. The majority of the flies spent at least some time on the rabbit with which they were confined. In the early trials (8, 29 and 52 h p.i.), many of the flies spent most of the 50 min period of confinement on the rabbits, usually around the eyes, mouth, nares and anus, and, almost uniformly, on the surface of the abdominal viscera after the abdominal cavity had been opened. Flies were also observed to spend long periods walking on the fur of the legs, feet and body of some rabbits. Following the modification of the procedure such that flies were removed immediately after they began feeding on an infected or uninfected control carcass, the flies were, in general, much more aggressive (i.e. likely to resume feeding) when they subsequently encountered the susceptible rabbits.

The results of the transmission trials are summarized in Table 3. None of the 5 rabbits held with flies that had been exposed to the uninoculated rabbit died. However, of the 10 susceptible rabbits that were held with potentially infected bushflies, 2 died of RHD, as confirmed by gross lesions and the RHD virus antigen-capture ELISA. The deaths occurred 5–6 days after exposure to the bushflies. In one case (no. 1266), the bushflies had initially been confined with the intact carcass of an infected rabbit (no. 876)

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Table 2. *Number of mosquitoes trapped at CO_2-baited traps on Wardang Island* (*June–October, 1995*)

Species trapped	Number trapped				
	June (8)*	July (1)	Aug. (2)	Sept. (4)	Oct. (2)
Aedes notoscriptus	2	0	0	0	0
Ae. camptorhynchus	1	22	7	21	0
Other†	0	0	1	2	0
Total	3	22	8	23‡	—

* Number of nights that traps were set up.
† Other includes: *Ae. vigilax*, *Culex australicus* and *Cx. globocoxitus*.
‡ Thirteen mosquitoes, 12 *Ae. camptorhynchus* and 1 *Ae. vigilax*, were trapped on one night.

Table 3. *Laboratory studies on the transmission of RHD virus with bushflies,* Musca vetustissima

Hours post-inoculation of primary rabbits	Primary rabbits	PCR on batches of flies (no. pos/no. tested)	Susceptible rabbits (no. dead/ no. exposed)
8	Inoculated	0/2	0/2
	Control	0/1	0/1
29	Inoculated	0/2	1/2
	Control	0/1	0/1
52	Inoculated	1/2	0/2
	Control	0/1	0/1
76	Inoculated	1/2	0/2
	Control	0/1	0/1
124	Inoculated	2/2	1/2
	Control	0/1	0/1
Total		4/15	2/15

at 29 h p.i.; in the other case (no. 1277), the flies had been confined with the opened carcass of the other infected rabbit (no. 871) at 124 h p.i. While the flies confined with no. 1266 were clearly observed on that rabbit for short periods, those confined with no. 1277 attacked it much more aggressively, consistent with the general change in fly behaviour that was observed with the modified protocol at 76 and 124 h p.i. In particular, flies persistently attacked no. 1277 around the eyes, nose and mouth, causing the rabbit to shake its head, blink vigorously and repeatedly, and even use its tongue to dislodge flies from around its mouth. Some flies were clearly observed with their proboscis extended into the conjunctival mucosa of this rabbit.

An RHD virus-specific PCR product was amplified in 4 of the 10 potentially infected batches of bushflies exposed to the inoculated rabbits (Table 3). None of the batches exposed to the uninfected control rabbit were positive by PCR. Of the 4 PCR-positive batches of flies, only 1 was associated with the death of a susceptible rabbit (no. 1277). Flies confined with the other susceptible rabbit that died (no. 1266) were negative for a specific PCR product.

Insect samples from RHD epizootics

Sixteen samples of insects, representing 5 species of flies and 3 species of mosquitoes, were chosen for analysis on the basis that they came from sites in south-eastern Australia where RHD was active. In 13 of the 16 samples, an RHD virus-specific product was amplified by PCR (Table 4). The only samples that were negative were: a very small sample of *M. vetustissima* and one of *C. dubia*, both from Wardang

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Table 4. *Details of 16 samples of insects collected at a number of epizootics of RHD in south-eastern Australia*

Sample	Location	Date	Insect species	PCR
1	Wardang Island	Sept. 95	*Musca vetustissima*	N
2	Wardang Island	Sept. 95	*Calliphora dubia*	N
3	Wardang Island	Sept. 95	*C. stygia*	P
4	Maldon	Apr. 96	*Aedes alboannulatus*	N
5	Maldon	Apr. 96	*Ae. postspiraculosis*	P
6	Maldon	Apr. 96	*Ae. notoscriptus*	P
7	Trevenson Park	Apr. 96	*Chrysomya rufifacies*	P
8	Trevenson Park	Apr. 96	*Ch. varipes**	P
9	Curtis 2	May 96	*Ch. rufifacies*	P
10	Curtis 2	May 96	*C. dubia*	P
11	Curtis 2	May 96	*M. vetustissima*	P
12	Curtis 2	May 96	*C. stygia*	P
13	Thackaringa	Nov. 95	*M. vetustissima*	P
14	Thackaringa	Nov. 95	*Ch. rufifacies*	P
15	Gum Creek	Nov. 95	*Musca* sp	P
16	Gum Creek	Nov. 95	*Chrysomyia* sp	P

* Following trituration of this sample of insects in PBS, 10% of the supernatant fluid was inoculated into a susceptible rabbit. The rabbit died 5 days post-inoculation.

Island; and, a sample of *Ae. alboannulatus* from Maldon, Victoria. One other species of fly from Wardang Island, and two other species of mosquito from Maldon were among those positive by PCR.

Following inoculation of the filtered supernatant fluid from each of the 16 samples of triturated insects into individual susceptible rabbits, only 1 rabbit died 5 days later. It had been inoculated with 2 ml of PCR-positive, supernatant fluid from a sample of *Ch. varipes* trapped at Trevenson Park, Victoria. None of the remaining PCR-positive samples killed rabbits.

DISCUSSION

The spread of RHD virus from the quarantine compound on Wardang Island, and eventually onto the Australian mainland, raised a number of questions concerning the transmission of the virus. Work on the island, and laboratory work by others [5, 9–11] had already suggested a number of possible mechanisms for the natural spread of RHD virus including: (1) direct rabbit to rabbit transmission following prolonged close contact between an infected rabbit or rabbit carcass and a susceptible rabbit; or (2) indirect spread either via contaminated burrows, insect vectors (e.g. fleas, mosquitoes and flies), or via avian and mammalian vectors. Recently [12], there has also been field evidence for the transmission of RHD virus by flies. Some form of airborne transmission was clearly possible to account for the spread of RHD virus from Wardang Island.

The data in Table 1 demonstrate that there was a general increase in fly activity on the island during early Spring (September). It was during this period, while the field trials were in progress, that RHD began to appear outside the experimental sites although still within the quarantine compound. Despite termination of the trials, and attempts to contain the spread of virus, RHD was found in wild rabbits outside the compound in early Spring, and, by mid-Spring, on the mainland at Point Pearce. Shortly after, the disease was confirmed at Yunta in South Australia, approximately 270 km northeast of Wardang Island. While increased activity was observed for 4 of the 5 fly species that were monitored, bushflies (*M. vetustissima*) showed the most pronounced rise and the highest absolute numbers, there being an approximate 1000-fold increase in the numbers trapped in September compared with the previous 3 months. High numbers were sustained through October when virus spread throughout the island and onto the mainland. Therefore, the data from these studies provide circumstantial, but compelling, evidence to support the hypothesis that flies, in particular the bushfly,

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M. vetustissima, may have a role as a vector of RHD virus.

Asgari and colleagues [12] have discussed aspects of the biology and feeding habits of flies of the genera *Calliphora* and *Chrysomya* that would allow them to be potential vectors of RHD virus. A number of factors make bushflies equally suitable candidates. Because they (1) feed naturally on both live and dead animals [13], (2) are able to penetrate 3–4 layers of cells with their mouthparts [14], and (3) regurgitate during or after biting [15], bushflies have the potential to transmit RHD virus between rabbits directly, or indirectly through contaminated flyspots [12]. Given that flies are known to move 7–15 km per day [13], it is feasible that bushflies could also have been responsible for spreading RHD, not only off the island to the mainland, but also to more distant points within Australia.

Six species of mosquitoes were initially identified on the island (*Aedes notoscriptus*, *Culex quinquefasciatus*, *Cx. annulirostris*, *Cx. australicus*, *Ae. australis* and *Ae. camptorhynchus*), and two more, *Ae. vigilax* and *Cx. globocoxitus*, were trapped in August and September. *Ae. camptorhynchus* is a recognized vector of myxomatosis [16]. Potential breeding areas of mosquitoes on the island were monitored regularly for larvae, and these sites were treated with *Bacillus thuringiensis* var. *israelensis* (VectoBac, Valent BioSciences, Libertyville, IL, USA) when necessary. Larvae were seldom seen once this treatment regime was established, and adult mosquitoes ceased to bite the staff carrying out treatments. As shown in Table 2, only small numbers of mosquitoes were caught in the CO_2/light traps on the island, although this may have been a reflection of the persistent wind on the island rather than evidence of a successful program to control mosquitoes. It is well known that mosquitoes are rarely trapped in windy conditions [17]. The two most successful catches were both on windless nights. There were insufficient data from trapping surveys to associate any increase in mosquito activity with the spread of RHD on, or from, Wardang Island.

Because there was circumstantial evidence to support the proposal that insects, particularly bushflies, were involved in the transmission of RHD virus, the hypothesis, that bushflies are capable of spreading the disease among rabbits, was then tested in laboratory experiments. Frequently, in this sort of trial insects are held in direct contact with, firstly, a source of infection, and then with the target host, often under highly controlled conditions (see for example [5, 9, 15]). However, in this study, transmission required that flies land on an infected rabbit or carcass, and, later, on a susceptible rabbit to transmit the disease. Although the experimental procedure was modified at 76 and 124 h p.i., the results (Table 3) indicated that both virus and disease could be transmitted by either procedure.

The data in Table 3 clearly show that, although a number of batches of flies were PCR-positive, not all of these transmitted RHD to susceptible rabbits. Given that there was, at the most, only 1 h between collection of flies exposed to infected rabbits and subsequent exposure of the flies to susceptible rabbits, it would seem unlikely that degradation of virus could account for this discrepancy, e.g. Asgari and colleagues [12] demonstrated that RHD virus lasted for up to 7 h on the legs of flies. Instead, the observation suggests that, under most circumstances, PCR is more sensitive than rabbit inoculation for detection of RHD virus. While Gehrmann and Kretzschmar [9] found that 10–100 virus particles was the minimum dose required to induce disease in rabbits, Guittre and colleagues [18] and Gould and colleagues [6] found that PCRs, based on a similar region of the genome, were capable of detecting 12 genome copies, and 5–10 copies per 100 mg of tissue, respectively, i.e. less than, or approximately equal to, the absolute minimum required to produce disease by rabbit inoculation.

Such a finding would be consistent with results from most of the laboratory and field work in this study. One exception, however, occurred in the laboratory work where a susceptible rabbit died of RHD following its exposure to a batch of flies that was negative by PCR for RHD virus (Table 3). While the reason for this inconsistent result is uncertain, one explanation might be that only 1 or 2 insects in the batch became contaminated with the minimum infectious dose of virus during contact with infected rabbit. Subsequent exposure of the susceptible rabbit to either of these flies could be sufficient to result in infection. However, with only 10% of the triturated suspension from the entire batch of flies being processed for PCR, it is possible that the amount of viral RNA in the small sample may have been below the limit of detection by PCR. The uniform absence of disease in susceptible control rabbits suggested that accidental iatrogenic transfer of RHD virus did not occur.

Laboratory results from this study indicate that both virus and disease can be transmitted from the carcass of an infected rabbit to a susceptible rabbit at

least 95 h after the death of the infected animal. The study also demonstrated that susceptible rabbits may die following exposure to flies that had been held with either intact or opened infected carcasses. These observations suggest a role for infected carcasses in the transmission of the natural disease regardless of whether the carcass is intact or whether the viscera have been exposed by a predator/scavenger. Since infectious virus may persist in the liver of infected carcasses for up to 3 weeks at 22 °C [19], it is possible that these carcasses could remain a source of infection, via vectors or directly, for at least the same period of time in the wild.

PCR examination of the 16 samples of insects from epizootics of RHD in south-eastern Australia showed that both flies and mosquitoes could become contaminated with RHD virus. However, only 1 of the 13 PCR-positive samples contained sufficient infectious virus to cause RHD when inoculated into susceptible rabbits. This may have been because, in most samples, the vast majority of virus had become degraded due to the long period that the contaminated insects spent in field traps prior to collection and analysis. An observation in support of this was that the only rabbit that died following inoculation survived for 5 days p.i. compared with the standard survival time of 36–48 h [5].

Only a single sample of bushflies from Wardang Island was examined for RHD virus, and it was negative by PCR and rabbit inoculation. It is possible that this was because the sample size of insects was small (only 26), and also because flies may have remained in the field trap too long (allowing degradation of flies, virus and viral RNA). Virus was, however, eventually identified in bushflies collected on the mainland (Table 4 [12]). Apart from the numerous species of flies found carrying RHD virus, this study also demonstrated that at least two species of mosquito carried RHD virus. This observation, together with the laboratory transmission trials of Lenghaus and colleagues [5], supports a role for mosquitoes in the epidemiology of RHD.

Two related questions that have important consequences for understanding transmission remain to be answered. First, do live infected rabbits excrete sufficient RHD virus to infect, or contaminate, insect vectors? In our laboratory experiment, flies were only exposed to live infected rabbits at 8 h p.i., when it was quite likely that the infected rabbits were not even excreting virus, and at 29 h p.i., when only one of the infected rabbits was still alive. Second, what is the maximum interval between infection, or contamination, of insect vectors, and transmission of infectious virus to a susceptible rabbit? In our study, 1 h was the only transfer time investigated, but more work is required to determine how long insects could carry infectious RHD virus. A further issue that remains to be addressed is whether bushflies are biological or mechanical vectors of RHD virus [20]. Work by Asgari and colleagues [12] suggests that the latter alternative is more likely.

Although Chasey [21] notes that there has been no reported evidence for the transfer of RHD virus over long distances by flies, the work in this study, together with the work of Asgari and colleagues [12], suggests that, within Australia, bushflies cannot be discounted as vectors, and that insect vectors in general are worth further investigation. In particular, investigations to assess whether insect vectors are capable of transmitting disease long distances over land or sea are necessary.

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