Novel Hendra Virus Variant Circulating in Black Flying Foxes and Grey-Headed Flying Foxes, Australia

Alison J. Peel,[1] Claude Kwe Yinda,[1] Edward J. Annand, Adrienne S. Dale, Peggy Eby, John-Sebastian Eden, Devin N. Jones, Maureen K. Kessler, Tamika J. Lunn, Tim Pearson, Jonathan E. Schulz, Ina L. Smith, Vincent J. Munster,[2] Raina K. Plowright[2]; Bat One Health Group[3]

A novel Hendra virus variant, genotype 2, was recently discovered in a horse that died after acute illness and in *Pteropus* flying fox tissues in Australia. We detected the variant in flying fox urine, the pathway relevant for spillover, supporting an expanded geographic range of Hendra virus risk to horses and humans.

Hendra virus (HeV; genus *Henipavirus*, family *Paramyxoviridae*) is a well-characterised zoonotic pathogen endemic to *Pteropus* spp. bats (flying foxes) in Australia. Spillover from bats to horses has been detected 65 times; 4 of 7 persons infected from horses have died (*1*). Quantitative reverse-transcription PCR (qRT-PCR) (*2*) is a tool used for surveillance and priority disease investigation in bats and horses (*3*,*4*). The high specificity of assays limits detection to a narrow range of genotypic diversity, meaning that divergent variants might remain undetected (*3*).

In October 2021, spillover of a novel variant, HeV genotype 2 (HeV-g2), resulted in the death of a horse in New South Wales (NSW), Australia, farther south than HeV had previously been detected in horses (*5*). This spillover was detected only because diagnostic assays had been recently updated after retrospective discovery of HeV-g2 in a horse that exhibited signs of HeV disease in 2015 but tested negative through routine screening at that time (*3*). Discovery of HeV-g2 in this horse arose using broad panparamyxovirus PCRs (*6*), followed by next-generation sequencing and virus isolation. The variant showed 84% pairwise nucleotide identity genome-wide to prototype HeV (HeV-g1), and 99% similarity with partial sequences recovered from tissue samples from a grey-headed flying fox, *P. poliocephalus* (*7*). Bats submitted for lyssavirus diagnostics were opportunistically screened using an updated quantitative PCR specific for HeV-g2, which resulted in additional positive detections in tissue collected from *P. poliocephalus* in 2019–2021 and a little red flying fox (*P. scapulatus*) in 2015 (*7*).

Although HeV-g1 has been detected in tissues from all 4 flying fox species in continental Australia, excretion of the virus has been confirmed only in the black flying fox (*P. alecto*) and the spectacled flying fox (*P. conspicillatus*), suggesting these species are sources of transmission to horses (*8*,*9*). Sequence mismatches between HeV-g1 and HeV-g2 mean that PCR assays used in previous surveillance of reservoir hosts would not have detected the novel HeV-g2. To address this gap, we used a new qRT-PCR (*3*) to screen banked flying fox urine samples collected over a large extent of space and time.

Author affiliations: Griffith University Centre for Planetary Health and Food Security, Nathan, Queensland, Australia (A.J. Peel, P. Eby, T.J. Lunn); National Institutes of Health, Hamilton, Montana, USA (C.K. Yinda, J.E. Schulz, V.J. Munster); EquiEpiVet, Aireys Inlet, Victoria, Australia (E.J. Annand); Department of Agriculture, Water, and the Environment, Canberra, Australian Capital Territory, Australia (E.J. Annand); University of Sydney, Sydney, New South Wales, Australia (E.J. Annand, J.-S. Eden); Texas Tech University, Lubbock, Texas, USA (A.S. Dale); University of New South Wales, Sydney (P. Eby); Montana State University, Bozeman, Montana, USA (D.N. Jones, M.K. Kessler, R.K. Plowright); Bellingen, New South Wales, Australia (T. Pearson); CSIRO, Black Mountain, Australian Capital Territory, Australia (I.L. Smith)

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[1]These authors contributed equally to this article.

[2]These senior authors contributed equally to this article.

[3]Members of Bat One Health are listed at the end of this article.

The Study

We collected pooled urine samples from plastic sheets placed underneath flying fox roosts in southeastern Queensland and mid- to north-coast NSW during December 2016–September 2020 (Figure). We placed sheets in areas of the roost where *P. alecto* flying foxes were roosting, although other species were often also present. We recorded the number and species of bats immediately above the sheets. We also captured individual bats in mist nests; recorded species, sex, and age class; then collected urine samples directly from each anaesthetised bat or from a urine collection bag attached to its holding bag. Shortly after collection, we placed samples into viral lysis buffer, virus transport media, or an empty cryovial and stored them at −80°C (Appendix, https://wwwnc.cdc.gov/EID/article/28/5/21-2338-App1.pdf).

We used the QIAamp Viral RNA Kit using a QIAcube HT automated system (QIAGEN, https://www.qiagen.com) to extract RNA, then eluted it in 150 μL of TE buffer and first screened it for HeV-g1 using a qRT-PCR assay targeting the P gene (Table 1). We stored extracted RNA at −80°C and then screened it for HeV-g2 using the new multiplexed qRT-PCR assay, targeting the M gene with primers specific for HeV-g1 and HeV-g2 (*2*,*3*) (Table 1; Appendix). We used 10-fold dilutions with a known number of genome copies to construct a standard curve, calculate copy numbers/mL, and estimate limit of detection. We amplified the partial cytochrome *b* gene from all positive samples (*10*,*11*) (Table 1) and confirmed host species identity based on sequence identity across 402-bp sequences (Appendix).

We screened 4,539 pooled urine samples collected from 129 underroost sampling sessions and 1,674 urine samples collected from individual bats over 39 catching sessions during July 2017–September 2020 (Appendix Tables 1, 2). Eight pooled urine samples and 2 samples from individual flying foxes tested positive for HeV-g2 (Table 2). Positive samples were from Sunnybank in Queensland and Clunes, Lismore, Dorroughby, Maclean, and Nambucca Heads in NSW.

We detected HeV-g2 in samples collected across all seasons. Prevalence in sessions with positive detections ranged from 2.5% to 6.5% (95% CI 0.1%–22.8%). In pooled samples, HeV-g2 was only detected in sessions when HeV-g1 was also detected (HeV-g1 prevalence range 2.5%–50.1%); however, we found no statistically significant correlation between HeV-g1 and HeV-g2 prevalence (Pearson correlation analysis ρ = 0.09; p = 0.87). Most (8/10) of the HeV-g2–positive samples had low genome copies, but 2, ARSUN015_15_1 and ARLIS002_55_1, had considerably higher copy numbers (Table 2).

Individual flying foxes that tested positive included a *P. poliocephalus* juvenile female captured in

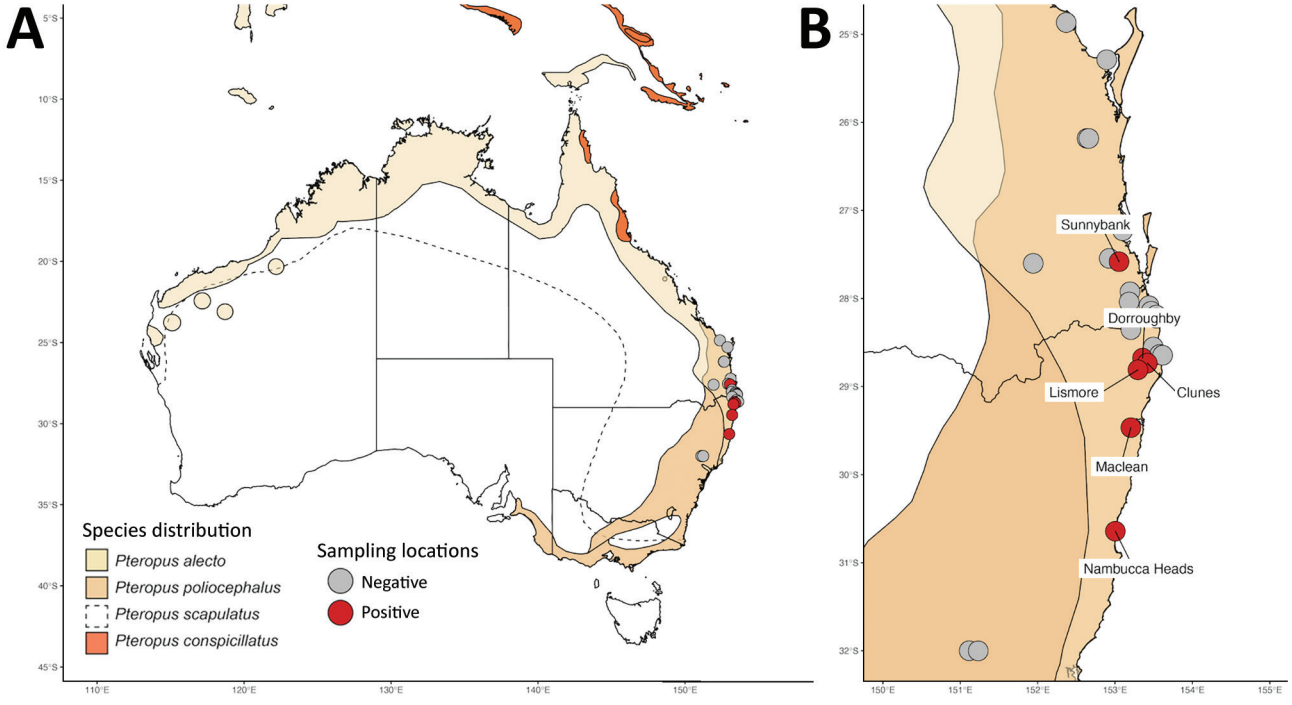

Figure. Distribution of flying fox species in Australia (*13*) and sampling locations for study of HeV variant circulating in flying foxes in southeastern Queensland and mid- to north-coast New South Wales, December 2016–September 2020. A) Locations in Australia; B) locations in study area. HeV, Hendra virus; HeV-g2, HeV genotype 2.

Table 1. Primers and probes used in PCR for study of novel Hendra virus variant circulating in black and grey-headed flying foxes, Australia*

Target	Primers and Probes	Reference
HeV-g1 P gene	F: 5′-CCCAACCAAGAAAGCAAGAG R: 5′-TTCATTCCTCGTGACAGCAC P: 5′-TTACTGCGGAGAATGTCCAACTGAGTG	This study
HeV-g1 M gene	F: 5′-CTTCGACAAAGACGGAACCAA R: 5′ TGGCATCTTTCATGCTCCATCTCGG P: 5′ CCAGCTCGTCGGACAAAATT	(*2*)
HeV-g2 M gene	F: 5′ TCTCGACAAGGACGGAGCTAA R: 5′ CCGGCTCGTCGAACAAAATT P: 5′ TGGCATCCTTCATGCTTCACCTTGG	(*3*)
Partial cytochrome b gene	F: 5′-CGAAGCTTGATATGAAAAACCATCGTTG R: 5′ AACTGCAGCCCCTCAGAATGATATTTGTCCTCA	(*10*,*11*)

*F, forward; R, reverse; P, probe.

Maclean, NSW, and a *P. alecto* adult male captured in Clunes, NSW (Appendix Table 3). We detected HeV-g2 in pooled samples from mixed-species roosts containing *P. alecto* and *P. poliocephalus* flying foxes. Cytochrome b sequencing identified DNA from *P. alecto* flying foxes in 6/8 positive underroost samples and from *P. poliocephalus* flying foxes in 2/8 (Table 2).

Conclusions

Urine is the route of HeV excretion from flying foxes and the source of virus transmission to horses. Detecting the novel Hendra variant HeV-g2 in the urine of flying foxes helped identify its distribution range, associated host species, transmission dynamics, and spillover risk. We show evidence that *P. alecto* and *P. poliocephalus* flying foxes excrete HeV-g2 in urine and both are likely competent reservoir hosts. We did not screen urine samples from *P. conspicillatus* or *P. scapulatus* flying foxes, so the potential of these species to excrete HeV-g2 in urine remains unconfirmed.

Although HeV-g1 has been detected in flying fox urine samples collected across all seasons, prevalence peaks in winter in subtropical regions (*4*,*12*), which is consistent with our preliminary HeV-g2 seasonality findings (5/8 detections in late May–late August) in the study area. The significantly lower prevalence of HeV-g2 than HeV-g1 could indicate actual lower prevalence in the sampled population. Alternatively, repeated freeze-thaw cycles in our samples or the bias toward collecting *P. alecto* urine in our sampling design might have led to lower detection. Tissue samples from flying foxes submitted for lyssavirus

Table 2. Details of urine samples collected from *Pteropus alecto* and *P. poliocephalus* flying foxes in underroost sampling sessions that tested positive for HeV-g2 and associated session-level prevalence for HeV-g1 and HeV-g2, Australia*

		HeV-g2		HeV-g1					
Site	Date	No. positive/ total	Prevalence, % (95% CI)	No. positive/ total	Prevalence, % (95% CI)	Sample ID	RNA copies/ mL†	Species recorded‡	*Cyt b* species§
Clunes, NSW	2019 Jul 27	1/36	2.8 (0.1–16.2)	0/36	0.0 (0–12.0)	ACMAC001_35_1	169	*Pa*	*Pa*
Maclean, NSW	2018 Jul 9	1/36	2.8 (0.1–16.2)	0/36	0.0 (0–12.0)	ACCLU004_22_1F	225	*Pp*	*Pp*
Clunes, NSW	2017 Aug 8	1/36	2.8 (0.1–16.2)	5/36	13.9 (5.2–30.3)	ACMAC001_35_1	174	2 *Pa*; 0 *Pp*	*Pa*
Clunes, NSW	2018 Nov 1	2/51	3.9 (0.7–14.6)	4/51	7.8 (2.5–19.7)	ARCLU002_14_1	38	0 *Pa*; 2 *Pp*	Mixed *Pp/Pa*
						ARCLU010_22_1	17	1 *Pa*; 2 *Pp*	*Pa*
Lismore, NSW	2017 Aug 27	1/48	2.1 (0.1–12.5)	21/48	43.8 (29.8–58.7)	ARCLU010_26_1	783	4 *Pa*; 0 *Pp*	NA
Nambucca Heads, NSW	2018 May 20	2/31	6.5 (1.1–22.8)	8/31	25.8 (12.5–50.1)	ARLIS002_55_1	67	0 *Pa*; 2 *Pp*	*Pa*
						ARNAM005_2_1	15	4 *Pa*; 0 *Pp*	*Pa*
Sunnybank, QLD	2018 Nov 26	1/36	2.8 (0.1–16.2)	1/36	2.8 (0.1–16.2)	ARNAM005_12_1	381,123	0 *Pa*; 4 *Pp*	*Pp*
Dorroughby, NSW	2016 Dec 16	1/18	2.5 (0.01–14.7)	1/18	2.5 (0.01–14.7)	ARSUN015_15_1	58	NR	*Pa*

**Cyt b, Cytochrome b*; HeV, Hendra virus; NSW, New South Wales; *Pa*, *P. alecto*; *Pp*, *P. poliocephalus*; QLD, Queensland; NA, not available; NR, not recorded.
†HeV-g2 viral copies/mL: the minimum copy number which would be expected to reliably give a positive PCR result in all replicates in the quantitative reverse transcription PCR assay (the limit of detection) was 5–10 copies per reaction (≈1,070–2,140 copies/mL).
‡For underroost samples, the number of flying foxes recorded by species (*P. alecto* or *P. poliocephalus*) at the time of sampling might not precisely reflect the proportion of urine collected from each species.
§Appendix Table 3 (https://wwwnc.cdc.gov/EID/article/28/5/21-2338-App1.pdf).

testing after contact with humans or pets showed higher HeV-g2 prevalence than our samples from wild populations (*7*), which might reflect higher prevalence in sick or stressed bats or geographical differences. HeV-g2 was previously detected in tissue samples from South Australia (3 positives from 4 samples), Victoria (7/64), and Western Australia (1/2) (*7*). Our findings extend the known distributional range of HeV-g2 to southeastern Queensland and mid- to north-coast NSW, areas proximate to the 2 known cases of HeV-g2 spillover to horses (*3*,*5*).

Our findings support expanding the expected geographic risk area for HeV spillover to include the distribution of *P. poliocephalus* flying foxes. Screening flying fox urine samples from a broader geographic range, including regions where *P. alecto* flying foxes are absent, should better inform epidemiologic relationships and relative prevalence of HeV variants. Given that data on the true diversity of HeV and related viruses in flying fox populations are incomplete, unbiased or *Paramyxoviridae* family–level viral surveillance in reservoir and spillover hosts might identify further variants. Developing a panel of diagnostic tools to detect a more comprehensive range of the viruses capable of spillover would substantially advance our ability to forecast spillover risk, manage biosecurity, and provide guidance to horse owners, veterinarians, and other stakeholders.

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About the Authors

Dr. Peel is a DECRA senior research fellow at Griffith University in Brisbane, Queensland, Australia. Her primary interests lie in the role of landscape change and anthropogenic influence on the dynamics and drivers of infectious disease in bats. Dr. Yinda is a postdoctoral research fellow at the Virus Ecology Section of the Rocky Mountains Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health. He is interested in pathogen discovery of emerging viruses, and their cross-species transmissions and disease potential.

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Address for correspondence: Alison Peel, Centre for Planetary Health and Food Security, Griffith University, Nathan Campus, Sir Samuel Griffith Centre (N78) 2.23, 170 Kessels Rd, Nathan, QLD 4111, Australia; email: a.peel@griffith.edu.au

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Appendix

METHODS

Sample Collection

Sample collection was covered under Griffith University Animal Ethics Committee Approval ENV 10 16 AEC.

Under-Roost Urine samples

Urine samples were collected from plastic sheets placed underneath flying fox roosts in southeast Queensland and mid- to north-coast New South Wales during December 2016–September 2020 (Figure 1). In each sampling session, up to 64 plastic sheets (0.9 m×1.3 m) were placed under roosting trees before dawn as decribed elsewhere (*1*). After bats had returned to the roost, a single pooled urine sample from each sheet was pipetted into a tube with AVL lysis buffer (QIAGEN; https://www.qiagen.com); the target amounts were 140 µL of urine into 560 µL buffer, 200–1000 µL of urine into 1000 µL of viral transport medium, or a plain cryovial. Sample collection occurred within approximately 7 hours of laying sheets (<6 h of sunrise). Samples were transferred to a CryoShipper (below –80°C) for transport and stored at –80°C in the laboratory. Species present within the roost at the time of sampling included black flying foxes (*Pteropus alecto*), grey-headed flying foxes (*P. poliocephalus*), and more rarely, little red flying foxes (*P. scapulatus*) (*2*). Where possible, sheet placements within the roosting area were prioritized towards where *P. alecto* and away from where *P. scapulatus* were roosting. The number and species of bats immediately above the sheet was recorded; however, in some cases individual bats were easily disturbed and took flight, meaning that these data are indicative only.

Samples from Captured Individuals

Urine samples were also collected directly from individual bats captured in mist nests at their roost site, using methods described elsewhere (Hansen et al., in review). Bats were held in cotton bags with the bottom third lined with plastic and a urine collection bag attached to facilitate the collection of samples. Bats were anaesthetised for further sample collection and their species, sex, and age class (adult, subadult, juvenile) were recorded. Urine samples were collected directly from the bat if it urinated while under anaesthetic, or from the urine collection bag. The former were prioritised for screening if both were available. Urine samples were placed into AVL buffer, viral transport medium or a plain cryovial and stored as described above.

Sample Selection

Over 10,000 urine samples were collected in combined under-roost and individual capture sessions. We selected a subset for Hendra virus genotype 2 (HeV-g2) testing using criteria that address the 2 main aims of the work: describing the distribution and dynamics of HeV-g2 in southeast Queensland and northeast New South Wales and exploring host species associations. Higher rates of detection were observed in under-roost sampling, as multiple individual bats above a sheet might contribute to each pooled sample (*3*). Screening samples collected using this approach maximizes the likelihood of detecting novel HeV-g2 variant if it is present. To explore distribution and dynamics, we screened 4,322 pooled urine samples collected from 127 under-roost sampling sessions during July 2017–September 2020. Samples were selected to represent the broad spatiotemporal coverage of the sample set (Appendix Table 1). To address host species associations, we selected samples attributed to either *P. alecto* or *P. poliocephalus*. Initially, we screened all available samples collected from individual bats where the species was identified in the field (674 urine samples collected from individual bats over 39 catching sessions during August 2017–September 2020, Appendix Table 2). Because the number of samples from captured bats was biased towards *P. alecto*, we included an additional 217 under-roost samples from 2 sessions with high proportions of *P. poliocephalus* (Maclean and Stewarts Brook, Appendix Table 1).

Cytochrome b Sequencing for Species Identification

Partial *cytochrome b* gene was amplified by PCR from all positive samples using previously described primers validated for species identification (Table 1). For amplification of *cytochrome b* DNA from each sample, 2 μL of complementary DNA template and primers at 0.2

μM each were used in a 25 μL reaction with the TopTaq master mix PCR premix (QIAGEN) and amplified with 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min. PCR products were run on 1% agarose gel and positive bands were excised and purified using NucleoSpin Gel and PCR Clean-up kit according to manufacturer instructions (MACHEREY-NAGE; https://www.mn-net.com). PCR products were Sanger sequenced (ACGT) and species confirmed based on >98% sequence identity across 402 bp length sequences. *Cytochrome b* sequences are listed in Appendix Table 3 below.

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Appendix Table 1. Detections of HeV-g2 by under-roost sampling sessions

Site	Start date	Positive	Total
Anchorage	2017 Aug 6	0	22
Anchorage	2017 Aug 31	0	26
Banora Green	2017 Aug 30	0	28
Burleigh	2018 Jul 1	0	36
Burleigh	2018 Jul 31	0	5
Burleigh	2018 Aug 31	0	9
Burleigh	2018 Nov 24	0	8
Burleigh	2019 Jan 3	0	32
Burleigh	2019 Jan 23	0	4
Burleigh	2019 Mar 6	0	4
Burleigh	2019 Mar 23	0	3
Burleigh	2019 Jul 26	0	3
Burleigh	2019 Oct 30	0	39
Burleigh	2020 Aug 24	0	9
Burleigh	2020 Sep 29	0	27
Byron Bay	2018 Jun 17	0	34
Canungra	2017 Aug 28	0	45
Canungra	2017 Sep 30	0	28
Canungra	2017 Oct 28	0	45
Canungra	2018 Jan 20	0	1
Clunes	2017 Jul 17	0	30
Clunes	2017 Aug 8	1	36
Clunes	2017 Sep 4	0	47
Clunes	2018 Feb 14	0	35
Clunes	2018 May 14	0	50
Clunes	2018 Jun 11	0	43
Clunes	2018 Aug 1	0	45
Clunes	2018 Aug 30	0	54
Clunes	2018 Oct 10	0	37
Clunes	2018 Nov 1	2	51
Clunes	2018 Nov 29	0	33
Clunes	2019 Jan 5	0	43
Clunes	2019 Jan 26	0	29
Clunes	2019 Feb 27	0	38
Clunes	2019 Mar 22	0	57
Clunes	2019 May 7	0	62
Clunes	2019 Jun 5	0	50
Clunes	2019 Jul 2	0	64
Clunes	2019 Jul 24	0	64
Clunes	2019 Aug 31	0	39
Clunes	2019 Sep 24	0	49
Clunes	2019 Oct 24	0	55
Clunes	2019 Nov 27	0	49
Clunes	2019 Dec 19	0	50
Clunes	2020 Jan 23	0	48
Clunes	2020 Feb 20	0	51
Clunes	2020 Mar 25	0	55
Clunes	2020 May 1	0	47
Clunes	2020 May 28	0	59
Clunes	2020 Jun 26	0	60
Clunes	2020 Aug 6	0	43
Clunes	2020 Aug 28	0	20
Clunes	2020 Sep 30	0	35
Commissioner's Gully	2020 Aug 25	0	7
Currumbin	2018 Oct 4	0	50
Dorroughby	2016 Dec 16	1	18
Gympie	2019 Jan 30	0	39
Hervey Bay	2018 Jul 23	0	26
Hervey Bay	2020 Jul 27	0	12
Lismore	2016 Dec 18	0	14
Lismore	2017 Aug 27	1	48
Lismore	2017 Oct 1	0	42
Lismore	2017 Oct 29	0	33
Lismore	2017 Nov 25	0	29
Lismore	2017 Dec 17	0	31
Lismore	2018 Sep 16	0	14
Lismore	2020 Aug 29	0	35
Maclean	2018 Jul 7	0	51

Site	Start date	Positive	Total
Mount Ommaney	2019 Jan 15	0	39
Mullumbimby	2017 Sep 5	0	37
Nambucca Heads	2017 Jul 26	0	28
Nambucca Heads	2017 Sep 2	0	35
Nambucca Heads	2017 Dec 17	0	15
Nambucca Heads	2018 Feb 11	0	18
Nambucca Heads	2018 May 20	2	31
Nambucca Heads	2018 Jul 22	0	17
Nambucca Heads	2018 Sep 2	0	16
Nambucca Heads	2018 Nov 25	0	8
Nambucca Heads	2019 Feb 17	0	15
Nambucca Heads	2019 Apr 28	0	20
Nambucca Heads	2019 Aug 13	0	37
Redcliffe	2018 Apr 27	0	30
Redcliffe	2018 May 24	0	51
Redcliffe	2018 Jun 29	0	56
Redcliffe	2018 Jul 26	0	57
Redcliffe	2018 Aug 28	0	43
Redcliffe	2018 Oct 3	0	32
Redcliffe	2018 Oct 31	0	33
Redcliffe	2018 Nov 30	0	36
Redcliffe	2019 Jan 4	0	27
Redcliffe	2019 Feb 14	0	30
Redcliffe	2019 Feb 28	0	23
Redcliffe	2019 Mar 25	0	43
Redcliffe	2019 Apr 29	0	35
Redcliffe	2019 Jul 9	0	41
Redcliffe	2019 Aug 2	0	26
Redcliffe	2019 Aug 30	0	47
Redcliffe	2019 Sep 27	0	50
Redcliffe	2019 Oct 25	0	35
Redcliffe	2019 Nov 29	0	54
Redcliffe	2019 Dec 20	0	1
Redcliffe	2020 Jan 24	0	38
Redcliffe	2020 Feb 26	0	52
Redcliffe	2020 Apr 3	0	36
Redcliffe	2020 Apr 24	0	31
Redcliffe	2020 May 22	0	39
Redcliffe	2020 Jun 24	0	33
Redcliffe	2020 Aug 5	0	51
Redcliffe	2020 Aug 26	0	52
Redcliffe	2020 Sep 23	0	1
Scone	2019 Jun 17	0	22
Stewarts Brook	2019 Jun 19	0	166
Simpson’s Creek	2017 Aug 23	0	49
Stokers Siding	2020 Jun 5	0	37
Stokers Siding	2020 Aug 1	0	26
Sunnybank	2017 Nov 23	0	7
Sunnybank	2018 Jan 22	0	4
Sunnybank	2018 Mar 1	0	2
Sunnybank	2018 Mar 13	0	1
Sunnybank	2018 Oct 27	0	11
Sunnybank	2018 Nov 26	1	36
Sunnybank	2019 Jan 2	0	52
Sunnybank	2020 Jan 29	0	27
Sunnybank	2020 Sep 25	0	33
Toowoomba	2018 Nov 27	0	48
Toowoomba	2018 Dec 23	0	46
Toowoomba	2019 May 2	0	46
Toowoomba	2019 Oct 29	0	13
Tyalgum	2019 Feb 9	0	48

Appendix Table 2. Detections of HeV-g2 in individual bats by capture sessions

Site	Start date	Positive	Total
Bundaberg	2020 Feb 5	0	15
Clunes	2017 Aug 12	0	17
Clunes	2018 Feb 19	0	1
Clunes	2018 Aug 2	0	13
Clunes	2019 Jul 25	1	36
Gympie	2019 Jan 31	0	32
Hervey Bay	2018 Jul 15	0	40
Hervey Bay	2020 Jul 28	0	59
Maclean	2018 Jul 9	1	36
Mount Ommaney	2019 Jan 17	0	42
Redcliffe	2018 May 25	0	47
Redcliffe	2018 Jul 27	0	51
Redcliffe	2018 Sep 14	0	54
Redcliffe	2018 Dec 14	0	49
Redcliffe	2019 Mar 8	0	47
Redcliffe	2019 May 28	0	31
Redcliffe	2019 Jul 9	0	50
Redcliffe	2019 Sep 10	0	49
Redcliffe	2019 Dec 3	0	51
Redcliffe	2020 Mar 3	0	48
Redcliffe	2020 May 11	0	63
Redcliffe	2020 Jul 7	0	53
Redcliffe	2020 Sep 7	0	52
Sunnybank	2018 Mar 16	0	36
Toowoomba	2018 Jun 3	0	52
Toowoomba	2018 Jul 21	0	52
Toowoomba	2018 Sep 8	0	55
Toowoomba	2018 Dec 8	0	61
Toowoomba	2019 Jan 11	0	8
Toowoomba	2019 Mar 15	0	49
Toowoomba	2019 May 14	0	54
Toowoomba	2019 Jul 2	0	56
Toowoomba	2019 Jul 23	0	4
Toowoomba	2019 Sep 3	0	57
Toowoomba	2019 Dec 10	0	48
Toowoomba	2020 Mar 10	0	54
Toowoomba	2020 May 4	0	54
Toowoomba	2020 Jul 14	0	47
Toowoomba	2020 Sep 1	0	46

Appendix Table 3. Cytochrome b sequences and species assessment for each sample

Sample ID	Cytochrome b sequence	HQ, %*	Assessment
ACCLU004_22_1F	TATTTCAACTACAAGAACCACAATGACAAACATCCGTAAATCACACCCAC TATTCAAAATTATCAACGACTCACTGATCGACCTACCCGCCCCATCAAGT ATTTCCTCATGATGAAACTTCGGCTCACTACTAGGCATCTGTCTAGCCAT CCAGATCCTAACAGGACTGTTCCTAGCTATACACTACACTTCAGACACAA CGACCGCCTTCCAATCCGTAACTCATATCTGCCGAGACGTAAACTACGGA TGAATCCTGCGTTATTTGCATGCTAACGGAGCATCCATATTCTTCATCTG CCTATTCTTACATGTAGGCCGAGGCCTCTACTACGGATCTTACATTTACA AAGAAACCTGAAACGTAGGTGTTATTCTCCTATTTGCTGTAATAGCAACA GCC	85.4	*Pteropus alecto* Cytb sequence has 100% match to *P. griseus* (KJ532423.1) and 98% match to *P alecto* (MN511367.1). This bat was captured in NSW and identified as *P. alecto* in the field. *P. griseus* is found in Indonesia but there is growing evidence from multiple studies (*4*,*5*) that *P. alecto* is poorly resolved relative to other species, and Australian populations include a newly recognised mitochondrial DNA lineage that suggests historical admixture with *P. griseus* (*4*). This individual was therefore assessed as *P. alecto*.
ACMAC001_35_1	TACTTCAACTACAAGAACCACAATGACAAACATCCGAAAATCACACCCAC TATTCAAAATTATCAACGACTCACTAGTCGACCTGCCCGCTCCATCAAGT ATCTCATCATGATGAAACTTTGGCTCACTACTAGGCATCTGCCTAGCCAT CCAAATCCTGACAGGACTGTTCCTAGCCATACACTACACTTCAGACACAA CAACCGCCTTCCAATCCGTGACTCACATCTGCCGAGACGTAAACTACGGA TGAATCCTCCGCTACTTACACGCTAACGGAGCATCCATATTCTTCATCTG CCTATTCCTACATGTGGGCCGAGGCCTCTACTACGGATCTTACATCTATA AAGAGACCTGAAACGTAGGTGTCATCCTTCTATTTGCCGTAATAGCAACA GCC	94.7	*P. poliocephalus* Cytb sequence has 99.48% match to *P. poliocephalus* (KJ532404.1). This bat was captured in NSW and identified as *P. poliocephalus* in the field.
ARCLU002_14_1	TACTTCAACTACAAGAACCACAATGACAAACATCCGCAAATCACACCCAC TATTCAAAGTTATCAACGACTCACTGATCGACCTACCCGCCCCATCAAGT ATTTCCTCATGATGAAACTTCGGCTCACTACTAGGCATCTGCCTAGCCAT CCAAATCCTAACAGGACTGTTCCTAGCTATACACTACACTTCAGACACAA CGACCGCCTTCCAATCCGTGACCCATATCTGCCGAGACGTAAACTACGGA TGAATTCTGCGTTATTTACATGCTAACGGAGCATCCATATTCTTCATCTG CCTATTCTTACATGTAGGCCGAGGCCTCTACTACGGATCTTATATCTACA AAGAAACCTGAAACGTAGGTGTTATTCTCCTATTTGCCGTAATAGCAACA GCC	94.6	*P. alecto* Cytb sequence has 99.48% match to *P. alecto* (KF726143.1). Two *P. alecto* were noted as roosting above the plastic sheet at the time of collection. No mixed peaks observed in chromatogram.
ARCLU010_22_1	TATTTCAACTACAAGAACCACAATGACAACCATCCGAAAATCACACCCCC TATTCAAAATAATCAACCACTCATTAGTCGACCTACCCGCTCCATCAAGT ATCTCATCATGATGAAACTTTGGCTCACTCCTAGGCATCTGCCTAACCAT CCAAATCACCACAGGACTGTTCCTAGCCATACACTACACTTCAGACACAT CAACCGCCTTTCAATCAATGACTCACATCAGCCGAGACGTAAATTACGGA TGAATCATCCGCTACTTACACGCCAACGGAGCATCCATATTCTTCATCTG CCTATTCCTACACATGGGCCGAGGCCTATATTACGGATCTTACATCTATA AAGAGACCTGAAACAGTAGCATCATCCTCCTATTTGCAGTAATAGCAACA GCC	33.0	Mixed *P. poliocephalus* and *P alecto* Cytb sequence has 92.67% match to *P. poliocephalus* (FJ561387.1), however it is a low-quality sequence due to multiple mixed peaks, where the alternative peak is consistent with *P. alecto* sequences. Two *P. poliocephalus* were noted as roosting above the plastic sheet at the time of collection. Suggestive of a mixed under-roost sample from *P. poliocephalus* *P. alecto*.
ARCLU010_26_1	TACTTCAACTACAAGAACCACAATGACAAACATCCGTAAATCACACCCAC TATTCAAAATTATCAACGACTCACTGATCGACCTACCCGCCCCATCAAGT ATTTCCTCATGATGAAACTTCGGCTCACTACTAGGCATCTGTCTAGCCAT CCAAATCCTAACAGGACTGTTCCTAGCCATACACTACACTTCAGACACAA CGACCGCCTTCCAATCCGTAACTCATATCTGCCGAGACGTAAATTACGGA TGAATCCTACGTTATTTGCATGCTAACGGAGCATCCATATTCTTCATCTG CCTATTCTTACATGTAGGCCGAGGCCTCTACTATGGATCTTACATTTACA AAGAAACCTGAAACGTAGGTGTTATTCTCCTATTCGCCGTAATAGCAACA GCC	78.4	*P. alecto* Cytb sequence has 98.16% match to *P. griseus* (KJ532423.1) and 95.78% match to *P. alecto* (KF726143.1). The sequence contained multiple mixed peaks, where the alternative peak is consistent with *P. alecto* sequences. Two *P. alecto* were noted as roosting above the plastic sheet at the time of collection. As described above for ACCLU004_22_1F, *P. alecto* is poorly resolved relative to other species, and include lineages closely related to *P. griseus*. Suggestive of a mixed under-roost sample from more than one *P. alecto*.
ARLIS002_55_1	TATTTCAACTACAAGAACACCAATGACCCCAATACGCAAAACTAACCCCC	94.0	*Homo sapiens*

Sample ID	Cytochrome b sequence	HQ, %*	Assessment
	TAATAAAATTAATTAACCACTCATTCATCGACCTCCCCACCCCATCCAAC ATCTCCGCATGATGAAACTTCGGCTCACTCCTTGGCGCCTGCCTGATCCT CCAAATCACCACAGGACTATTCCTAGCCATGCACTACTCACCAGACGCCT CAACCGCCTTTTCATCAATCGCCCACATCACTCGAGACGTAAATTATGGC TGAATCATCCGCTACCTTCACGCCAATGGCGCCTCAATATTCTTTATCTG CCTCTTCCTACACATCGGGCGAGGCCTATATTACGGATCATTTCTCTACT CAGAAACCTGAAACATCGGCATTATCCTCCTGCTTGCAACTATAGCAACA GCC		Cytb sequence has 100% match to *Homo sapiens*, indicating the sample was likely contaminated.
ARNAM005_2_1	TACTTCAACTACAAGAACCACAATGACAAACATCCGTAAATCACACCCAC TATTCAAAATTATCAACGACTCACTGATCGACCTACCCGCCCCATCAAGT ATTTCCTCATGATGAAACTTCGGCTCACTACTAGGCATCTGTCTAGCCAT CCAGATCCTAACAGGACTGTTCCTAGCCATACACTACACTTCAGACACAA CGACCGCCTTCCAATCCGTAACTCATATCTGCCGAGACGTAAATTACGGA TGAATCCTACGTTATTTGCATGCTAACGGAGCATCCATATTCTTCATCTG CCTATTCCTACATGTAGGCCGAGGCCTCTACTATGGATCTTACATTTACA AAGAAACCTGAAACGTAGGTGTTATTCTCCTATTCGCCGTAATAGCAACA GCC	95.7	*P. alecto* Cytb sequence has 98.51% match to *P. alecto* (KF726143.1). Although two *P. poliocephalus* were noted as roosting above the plastic sheet at the time of collection, sequence data is suggestive of a sample from *P. alecto*.
ARNAM005_12_1	TACTTCAACTACAAGAACCACAATGACAAACATCCGCAAATCACACCCAC TATTCAAAATTATCAACGACTCACTGATCGACCTACCCGCCCCATCAAGT ATTTCCTCATGATGAAACTTCGGCTCACTACTAGGCATCTGCCTAGCCAT CCAAATCCTAACAGGACTATTCCTAGCCATACACTACACTTCAGACACAA CGACCGCCTTCCAATCCGTGACCCATATCTGCCGAGACGTAAATTACGGA TGAATTCTGCGTTATTTACATGCTAACGGAGCATCCATATTCTTCATCTG CCTATTCTTACATGTAGGCCGAGGCCTCTACTACGGATCTTACATCTACA AAGAAACCTGAAACGTAGGTGTTATTCTCCTATTTGCCGTAATAGCAACA GCC	55.6	*P. alecto* Cytb sequence has 98.51% match to *P. alecto* (KF726143.1). Low-quality sequence includes non-diagnostic mixed peaks. Four *P. alecto* were noted as roosting above the plastic sheet at the time of collection.
ARSUN015_15_1	TACTTCAACTACAAGAACCACAATGACAAACATCCGAAAATCACACCCAC TATTCAAAATTATCAACGACTCACTAGTCGACCTACCCGCTCCATCAAGT ATCTCATCATGATGAAACTTTGGCTCACTACTAGGCATCTGCCTAGCCAT CCAAATCCTGACAGGACTGTTCCTAGCCATACACTACACTTCAGACACAA CAACCGCCTTCCAATCCGTGACTCACATCTGCCGAGACGTAAACTACGGA TGAATCCTCCGCTACTTACACGCTAACGGAGCATCCATATTCTTCATCTG CCTATTCCTGCATGTGGGCCGAGGCCTCTACTACGGATCTTACATCTATA AAGAGACCTGAAACGTAGGTGTCATCCTTCTATTTGCCGTAATAGCAACA GCC	96.2	*P. poliocephalus* Cytb sequence has 99.48% match to *P. poliocephalus* (KJ532404.1). Four *P. poliocephalus* were noted as roosting above the plastic sheet at the time of collection
ARDOR001_S2_1	TACTTCAACTACAAGAACCACAATGACAAACATCCGCAAATCACACCCAC TATTCAAAATTATCAACGACTCACTGATCGACCTACCCGCCCCATCAAGT ATTTCCTCATGATGAAACTTCGGCTCACTACTAGGCATCTGCCTAGCCAT CCAAATCCTAACAGGACTATTCCTAGCTATACACTACACTTCAGACACAA CGACCGCCTTCCAATCCGTGACCCATATCTGCCGAGACGTAAATTACGGA TGAATTCTGCGTTATTTACATGCTAACGGAGCATCCATATTCTTCATCTG CCTATTCTTACATGTAGGCCGAGGCCTCTACTACGGATCTTATATCTACA AAGAAACCTGAAACGTAGGTGTTATTCTCCTATTTGCCGTAATAGCAACA GCC	96.1	*P. alecto* Cytb sequence has 100% match to *P. alecto* (KJ532406.1) and *P. conspicillatus* (KJ532443.1). *P. conspicillatus* are not present in NSW and *P. alecto* were noted at the roost at the time of collection. Sequence data is suggestive of a sample from P. alecto.

*The percentage of bases in a sequence that are high quality (HQ) in the sequence.