DOI: 10.7589/2016-05-100

Journal of Wildlife Diseases, 53(1), 2017, pp. 111–120

PHYSIOLOGIC BIOMARKERS AND HENDRA VIRUS INFECTION IN AUSTRALIAN BLACK FLYING FOXES (*PTEROPUS ALECTO*)

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ABSTRACT: Bats of the genus *Pteropus* (Pteropodidae), colloquially known as flying foxes, are recognized as the natural reservoir of Hendra virus, a zoonotic paramyxovirus responsible for mortality in horses and humans. Some previous studies have suggested that physiologic and ecologic factors promote Hendra virus infection in flying foxes, and by extension, spillover to horses and humans. However, the impact of Hendra virus infection on relevant physiologic biomarkers in flying foxes has not been measured. Over 12 mo in eastern Australia, we captured and sampled 446 individual black flying foxes (*Pteropus alecto*), a putative primary reservoir host species, and measured a suite of hematologic, plasma biochemistry, and urinary biomarkers. All mean hematologic and biochemical values in both Hendra virus–positive and virus–negative cohorts were within the published reference ranges for black flying foxes. We found no association between Hendra virus infection (as indicated by PCR detection of Hendra virus RNA) and biomarkers for nutritional stress, reproductive stress, or extreme metabolic demand. However, we identified associations between several other biomarkers and Hendra virus infection, which may partly elucidate the physiologic effects of Hendra virus infection in flying foxes. Our findings highlight the need for critical evaluation of putative risk factors for infection in flying foxes and provide insights for future epidemiologic studies of Hendra virus and related viruses in the *Pteropus* species.

Key words: Biochemistry, black flying fox, hematology, Hendra virus, *Pteropus alecto,* risk factor.

INTRODUCTION

In Australia, bats of the genus *Pteropus* (Pteropodidae), colloquially known as flying foxes, have been identified as the natural reservoir of Hendra virus, a novel zoonotic agent responsible for morbidity and mortality in horses and humans (Murray et al. 1995; Halpin et al. 2000). Of the four species endemic to mainland Australia, the black (*Pteropus alecto)* and spectacled (*Pteropus conspicillatus*) flying foxes are the evident primary reservoir hosts (Edson et al. 2015; Field et al. 2015). It has been suggested that flying foxes and Hendra virus (and more broadly, bats and henipaviruses) have co-evolved and that the consequent mature host-agent relationship results in an absence of overt clinical disease associated with infection (Halpin et al. 2011). However, to date, the impact of Hendra virus infection on relevant physiologic biomarkers in flying foxes has not been measured.

Studies suggest that various physiologic factors (including stress associated with reproduction and suboptimal nutrition) may be positively associated with Hendra virus infection in flying foxes (Plowright et al. 2008; Breed et al. 2011), and by extension, with spillover into horses and humans (Plowright et al. 2015). However, biologic indicators of physiologic stress are difficult to measure directly, incorporating factors as diverse as immune function, endocrine function, neurologic function, nutrition, reproduction, environmental conditions, and social interactions (Bartolomucci 2007; Busch and Hayward 2009; Martin 2009). Instead, the measurement of physiologic biomarkers (hematologic, biochemical, and urine parameters) offers a

practical and effective alternative to direct measurement of biologic indicators and is routinely used in human and veterinary medicine (McLaughlin et al. 2007; Hall et al. 2014). Reference ranges for hematologic and biochemical biomarkers in black flying foxes, as well as attributed seasonal variation, are available (McMichael et al. 2015; McMichael et al. 2016). In this study, we examine the relationship between Hendra virus infection status and physiologic biomarkers and discuss the findings in the context of putative physiologic and environmental risk factors for Hendra virus infection in black flying foxes.

MATERIALS AND METHODS

Animals, ethics, and study sites

Blood, urine, and swab samples were collected from individual wild-caught black flying foxes during seven bimonthly catching events at a periurban parkland roost at Boonah, Southeast Queensland, Australia (27°59′S, 152°40′E) from June 2013 to May 2014. The study was a discrete component of a larger epidemiologic study of Hendra virus disease ecology reported elsewhere (Edson et al. 2015). Fieldwork was conducted under the Queensland Department of Agriculture, Fisheries and Forestry Animal Ethics Committee Permit SA 2011/12/375 and the Queensland Department of Environment, Heritage and Protection Scientific Purposes Permits WISP05810609 and WISP14100614.

Animal capture and sampling

Bats were captured predawn as per McMichael et al. (2015) and anesthetized (under veterinary supervision) by using the inhalation agent isoflurane and medical oxygen, as described by Jonsson et al. (2004). Postcapture sampling was random. Sex and age-class were recorded for each bat, with the latter using forearm length (millimeters), weight (grams), and presence of secondary sexual characteristics (Epstein et al. 2008). We collected 3 mL of blood (approximately 0.5% total blood volume) from the propatagial (cephalic) vein and dispensed into a 1.3-mL lithium heparin blood tube (Sarstedt 41.1393.105, Sarstedt, Nümbrecht, Germany), a 0.5 mL ethylenediaminetetraacetic acid tube (Microtainer 5974, Becton Dickinson, Franklin Lakes, New Jersey, USA), and a 1.0-mL serum tube (Sarstedt 41.1392.005, Sarstedt). All blood samples were collected within 6 h of capture. Urine samples were obtained by gentle manual bladder expression. Nasal, oral, rectal, and urogenital (females) or preputial (males) swabs were placed into 0.5 mL of phosphate-buffered saline. After anesthesia, each bat was monitored until conscious and hemostasis at the venipuncture site was confirmed prior to placing the bat into a suspended pillowcase to fully recover for at least 30 min before release at the capture site.

Hematologic, biochemical, and urine analysis

Blood samples were submitted to the accredited Queensland Medical Laboratory for hematology and plasma biochemistry; blood glucose concentration was measured at the time of bleeding by using an Accu-Chek Performa glucometer (Roche Diagnostics GmbH, Mannheim, Germany) according to McMichael et al. (2015). Urinalysis was conducted at the field site within 4 h of urine collection by using Urispec Plus reagent test strips (Henry Schein Animal Health, Dublin, Ohio, USA). Each urine sample was ascribed the highest semiquantitative value for the corresponding color reaction of the test strip or intensity upon visual assessment. Urine specific gravity (USG) was measured by using a handheld clinical refractometer.

Hendra virus detection

Serum and urine samples from all animals were screened for Hendra virus RNA, as described in the following. Animals found positive or equivocal on at least one sample had their full complement of samples screened for Hendra virus RNA. Where a urine sample was not obtained, the urogenital or prepucial swab was tested as a proxy. We added 50 µL of each serum, urine, and swab-extract sample to a corresponding 130 µL of Magmax lysis buffer (AM 8500, Ambion, Austin, Texas, USA) for Hendra virus PCR analysis at the Queensland Government Biosecurity Sciences Laboratory and stored at –80 C prior to testing. Samples underwent total nucleic acid extraction at the Queensland Government Biosecurity Sciences Laboratory Physical Containment Level 3 Laboratory by using the KingFisher automated extraction system (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the MagMax Viral RNA Isolation Kit (AMB18365, Ambion) according to manufacturer's instructions. We added 5 µL of RNA extract to 20 µL of TaqMan Real-Time PCR reaction mix (Ambion) and assayed them in duplicate for Hendra virus RNA by using a quantitative TaqMan Real-Time PCR targeting the Hendra virus M gene (Smith et al. 2001) in an Applied Biosystems 7500 Fast Real Time PCR System (Thermo Fisher Scientific). The real-time PCR results were analyzed by AB7500 software version 2.0.6 (Applied Biosystems, Thermo Fisher

Scientific). A mean cycle threshold value of <40 was defined as a positive result.

Statistical analysis

We used generalized linear models (GLM) to test the association between Hendra virus infection status and 18 hematologic, 22 biochemical, and eight urinary biomarkers. Hendra virus infection status was determined by the presence or absence of Hendra virus RNA in urine, serum, and swab matrices. The Hendra virus–positive cohort consisted of animals that were RNA positive in any sample type. The Hendra virus–negative cohort included animals that were RNA negative on all samples. Animals that had a negative urogenital or prepucial swab taken in the absence of a urine sample were excluded from the negative cohort to avoid potentially false-negative individuals, as the diagnostic sensitivity of such swabs is less than that of urine (Edson et al. 2015).

When values for hematologic or biochemical biomarkers were lower than the detectable limit of the assay, we adopted the recommended practice of assigning half of the detectable concentration for the comparative analyses (Wood et al. 2011). Data that proved to be positively skewed with heterogeneous variance were transformed by using the natural log. Each hematologic, biochemical, and urinary biomarker was subjected to an unbalanced GLM (McCullagh and Nelder 1989) under the normal or log-normal distribution as appropriate for continuous variables and the binomial distribution and logit link for binary variables, using GenStat (VSNi 2015). The residual plots for most biomarkers were approximately normally distributed. Adjusted means and standard errors were estimated for each biomarker (standardized for adult animals) for month+sex+sex by month interaction+Hendra virus–positive animal.

Biomarkers were then grouped into appropriate biologic panels: erythrocytes, leukocytes, electrolytes, metabolites and enzymes, nutrition, and urine (Table 1) and were subjected to a principal component analysis (PCA; Mardia et al. 1979) comparing Hendra virus status, gender, and season (spring-summer and autumn-winter).

RESULTS

Samples were collected from 446 individual black flying foxes; however, a full suite of samples was not collected from every bat. Immature age-class bats were excluded from analysis because of the limited sample size (n=67). In total, 241 whole blood samples, 234 plasma samples, and 251 urine samples from mature age-class (adult) animals were submitted for hematologic analysis, biochemical analysis, and urinalysis, respectively.

Hendra virus RNA was detected in 30 animals, with 27 positive in urine or urogenital or prepucial swabs, two positive in serum only, and one positive in nasal swab only (Edson et al. 2015). Blood and urine from all 30 Hendra virus–positive bats were submitted for hematologic, biochemical and urine analysis. A total of 236 Hendra virus–negative animals (a maximum of 50 per sampling event) were submitted for hematologic, biochemical, and urine analysis.

All mean hematologic and biochemical biomarker values in both Hendra virus–positive and Hendra virus–negative cohorts were within the published reference ranges for adult black flying foxes (McMichael et al. 2015). Results from the Hendra virus GLM analysis are reported in Table 1.

The presence of Hendra virus RNA was significantly associated with higher lymphocyte percentage, plasma alkaline phosphatase levels, urinary protein levels, and lower neutrophil percentage, and plasma triglyceride levels (Fig. 1). There were also nonsignificant trends toward higher mean lymphocyte counts, erythrocyte mean cell volume, urinary pH, lower mean platelet counts, and plasma potassium levels in Hendra virus–positive animals.

The PCA demonstrated seasonal changes for all biomarker panels for females, while males demonstrated seasonal changes for the metabolite and enzyme biomarker panel only (Supplementay Material Fig. 1). The Hendra virus–positive cohort was indistinguishable from the Hendra virus–negative cohort for both gender and season for the majority of the panels. However, for the leukocyte panel, the Hendra virus–positive cohort demonstrated a subtle right-shift trend compared with the Hendra virus–negative cohort for females across both seasons and for males in summer (Fig. 2).

DISCUSSION

We examined the impact of Hendra virus infection on physiologic biomarkers in flying

TABLE 1. Comparison of generalized linear model–generated mean hematologic, biochemical, and urinalysis biomarker values and principal component analysis (PCA) biomarker panel assignment for Hendra virus (HeV)–positive and HeV–negative cohorts of the putative HeV reservoir, the black flying-fox (*Pteropus alecto*), sampled in Australia, 2013–14.

Biomarker	HeV positive Mean (SE)	HeV negative Mean (SE)	*P*	PCA panel
Hematology				
Hemoglobin (g/L)	161.00 (2.77)	163.20 (0.73)	0.447	Erythrocyte
Red cell count ($\times 10^{12}$/L)	8.86 (0.16)	9.13 (0.04)	0.108	Erythrocyte
Hematocrit	0.47 (0.01)	0.47 (0.00)	0.696	Erythrocyte
Mean corpuscular volume (fL)	52.78 (0.65)	51.61 (0.17)	0.083	Erythrocyte
Mean corpuscular hemoglobin (pg)	18.23 (0.20)	17.94 (0.05)	0.175	Erythrocyte
Mean corpuscular hemoglobin concentration (g/L)	344.90 (1.58)	347.4 (0.42)	0.124	Erythrocyte
Platelets ($\times 10^9$/L)	320.90 (23.93)	366.90 (6.35)	0.064	No panel
Total leukocyte count ($\times 10^9$/L)	6.65 (0.69)	6.46 (0.18)	0.797	Leukocyte
Neutrophil count ($\times 10^9$/L)	3.49 (0.43)	3.94 (0.11)	0.310	Leukocyte
Lymphocyte count ($\times 10^9$/L)	2.82 (0.40)	2.12 (0.11)	0.087	Leukocyte
Monocyte count ($\times 10^9$/L)	0.13 (0.05)	0.19 (0.01)	0.352	Leukocyte
Eosinophil count ($\times 10^9$/L)	0.21 (0.12)	0.22 (0.03)	0.885	Leukocyte
Basophil count ($\times 10^9$/L)	0.00 (0.00)	0.00 (0.00)	0.756	Leukocyte
Neutrophil (%)	**53.78 (3.99)**[a]	**62.49 (1.06)**	**0.035**	No panel
Lymphocyte (%)	**40.81 (3.46)**	**31.84 (0.92)**	**0.013**	No panel
Monocyte (%)	2.02 (0.62)	2.74 (0.16)	0.257	No panel
Eosinophil (%)	3.38 (1.13)	2.88 (0.30)	0.667	No panel
Basophil (%)	0.01 (0.06)	0.04(0.02)	0.679	No panel
Biochemistry				
Sodium (mmol/L)	137.90 (2.28)	140.5 (0.73)	0.279	Electrolyte
Chloride (mmol/L)	103.80 (1.92)	106.8 (0.61)	0.140	Electrolyte
Potassium (mmol/L)	3.77 (0.18)	4.10 (0.06)	0.083	Electrolyte
Phosphorous (mmol/L)	1.50 (0.10)	1.45 (0.03)	0.599	Electrolyte
Calcium (mmol/L)	2.44 (0.05)	2.44 (0.01)	0.988	Electrolyte
Bicarbonate (mmol/L)	15.13 (0.50)	14.96 (0.16)	0.760	Electrolyte
Anion gap (mmol/L)	22.79 (0.65)	22.79 (0.21)	0.998	Electrolyte
Urea (mmol/L)	2.28 (0.31)	1.87 (0.10)	0.200	Metabolite/enzyme
Creatinine (μmol/L)	39.85 (4.46)	43.51 (1.43)	0.437	Metabolite/enzyme
Bilirubin (μmol/L)	1.23 (0.24)	1.27 (0.08)	0.863	Metabolite/enzyme
Aspartate aminotransferase (U/L)	57.46 (1.11)	65.76 (1.04)	0.238	Metabolite/enzyme
Alanine aminotransferase (U/L)	18.32 (1.10)	16.78 (1.03)	0.369	Metabolite/enzyme
Gamma-glutamyl transferase (U/L)	5.19 (1.10)	5.26 (1.03)	0.890	Metabolite/enzyme
Alkaline phosphatase (U/L)	**522.20 (43.18)**	**431.00 (13.78)**	**0.046**	Metabolite/enzyme
Creatinine kinase (U/L)	189.24 (1.24)	237.94 (1.07)	0.313	Metabolite/enzyme
Total protein (g/L)	66.44 (1.43)	66.16 (0.47)	0.854	Nutrition
Albumin (g/L)	36.86 (0.83)	36.62 (0.27)	0.779	Nutrition
Globulin (g/L)	29.49 (0.89)	29.49 (0.29)	0.998	No panel
Albumin:globulin ratio	1.29 (0.04)	1.28 (0.01)	0.814	No panel
Glucose (mmol/L)	6.62 (0.35)	6.95 (0.08)	0.351	Nutrition
Cholesterol (mmol/L)	0.40 (0.05)	0.39 (0.02)	0.839	Nutrition
Triglycerides (mmol/L)	**0.15 (0.04)**	**0.25 (0.01)**	**0.022**	Nutrition

TABLE 1. Continued.

Biomarker	HeV positive Mean (SE)	HeV negative Mean (SE)	*P*	PCA panel
Urinalysis				
Urine specific gravity	1.017 (0.00)	1.017 (0.00)	0.950	Urine
Ketones (mg/dL)	140.3 (20.55)	140.1 (4.94)	0.991	Urine
pH	7.51 (0.24)	7.08 (0.06)	0.073	Urine
Protein (mg/dL)	**9.87 (1.44)**	**3.90 (1.09)**	**0.013**	Urine
Glucose (mg/dL)	4.17 (1.47)	3.25 (1.10)	0.524	Urine
Blood (erythrocyte/μL)	2.75 (1.39)	2.40 (1.08)	0.691	Urine
Nitrite (mg/dL)	1.01 (1.02)	1.01 (1.01)	0.836	Urine
Leukocytes (leukocyte/μL)	6.33 (1.41)	4.96 (1.08)	0.491	Urine

[a] Analytes that significantly ($P<0.05$) differed between cohorts are shown in bold print.

foxes. Our use of GLM, to analyze and adjust individual biomarker data for life cycle and seasonal variations, and PCA, to analyze biologically plausible biomarker panels comparing gender and season, provided for the statistical robustness of our findings. The key finding that all mean hematologic and biochemical values in both the Hendra virus–positive and the Hendra virus–negative cohorts were within published reference ranges (McMichael et al. 2015) indicated that natural Hendra virus infection in black flying foxes does not routinely cause overt disease. Nonetheless, we found statistically significant differences in several biomarkers when comparing the cohorts. We discuss the findings in the context of putative physiologic and environmental risk factors for Hendra virus infection in *P. alecto*, mindful that statistical significance does not necessarily equate to biologic significance.

Hematology

All mean leukocyte values were within the published reference ranges, indicating that current or recent Hendra virus infection (demonstrated by the detection of viral RNA) does not provoke a strong immune response in black flying foxes, at least in terms of significant changes in leukocyte counts. This finding is consistent with the accumulated epidemiologic evidence that the black flying fox is a key reservoir species for Hendra virus (Edson et al. 2015; Field et al. 2015) and the broader recognition that viral infections of natural reservoir host species are commonly subclinical (Miller and Murray 2015).

The absence of a "stress leukogram" response (lymphopenia, eosinopenia, neutrophilia) in the Hendra virus–positive cohort argues against physiologic stress as a determinant of Hendra virus infection in black flying foxes. McMichael et al. (2015) demonstrated an expected stress leukogram response in female black flying foxes consistent with parturition, although it is unclear how readily this species would produce such a response to other acute physiologic or chronic ecologic stressors. Further, the absence of a decreased total leukocyte count suggests that immune suppression (Davis et al. 2008) is not a feature of Hendra virus infection in black flying foxes. We also found no evidence of an association between Hendra virus infection and hematologic biomarkers indicative of bacterial or parasitic infection. Elevated neutrophil or monocyte counts, reported by McMichael et al. (2015) to be indicative of bacterial infection in black flying foxes, were absent, as were elevated eosinophil or basophil counts, reported by Weber et al. (2002) and Opara and Fagbemi (2008) to be associated with parasite loads in wildlife species.

Although the PCA demonstrated only a subtle difference between the leukocyte biomarker panel values between the Hendra virus–positive and virus–negative cohorts, the GLM demonstrated a significantly higher lymphocyte percentage and concomitant low-

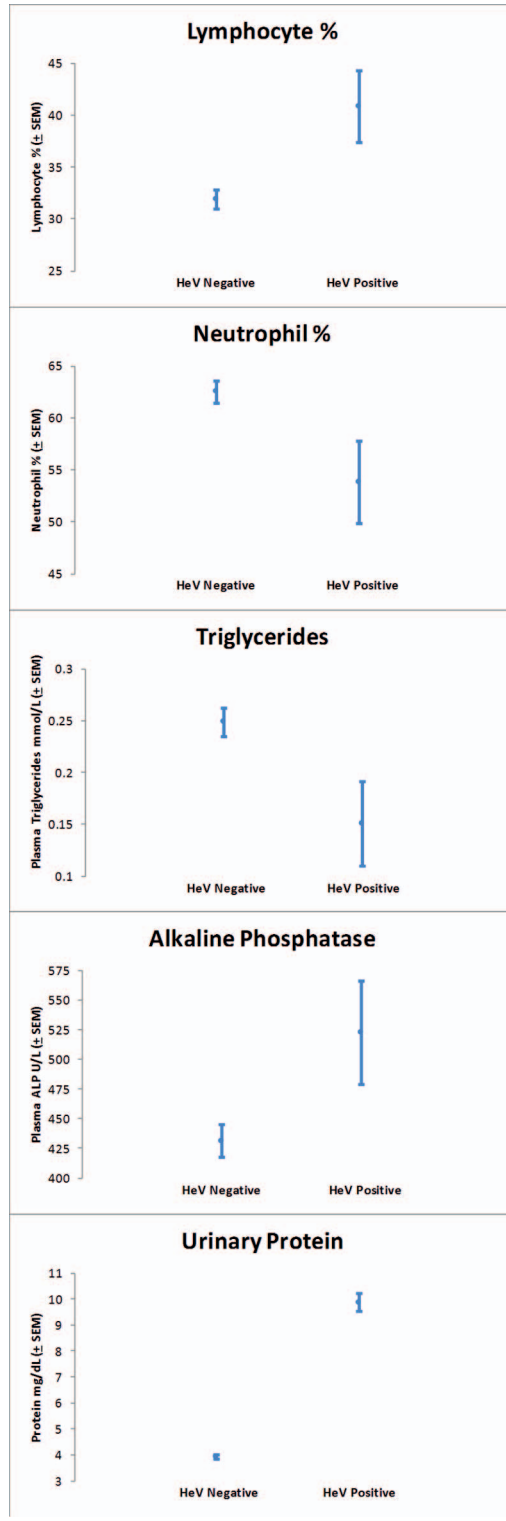

FIGURE 1. Statistically significant differences between generalized linear model–generated Hendra virus (HeV)–positive and HeV–negative cohort mean hematologic, biochemical, and urine biomarker values of samples taken from black flying foxes (*Pteropus alecto*) in Australia, 2013–14.

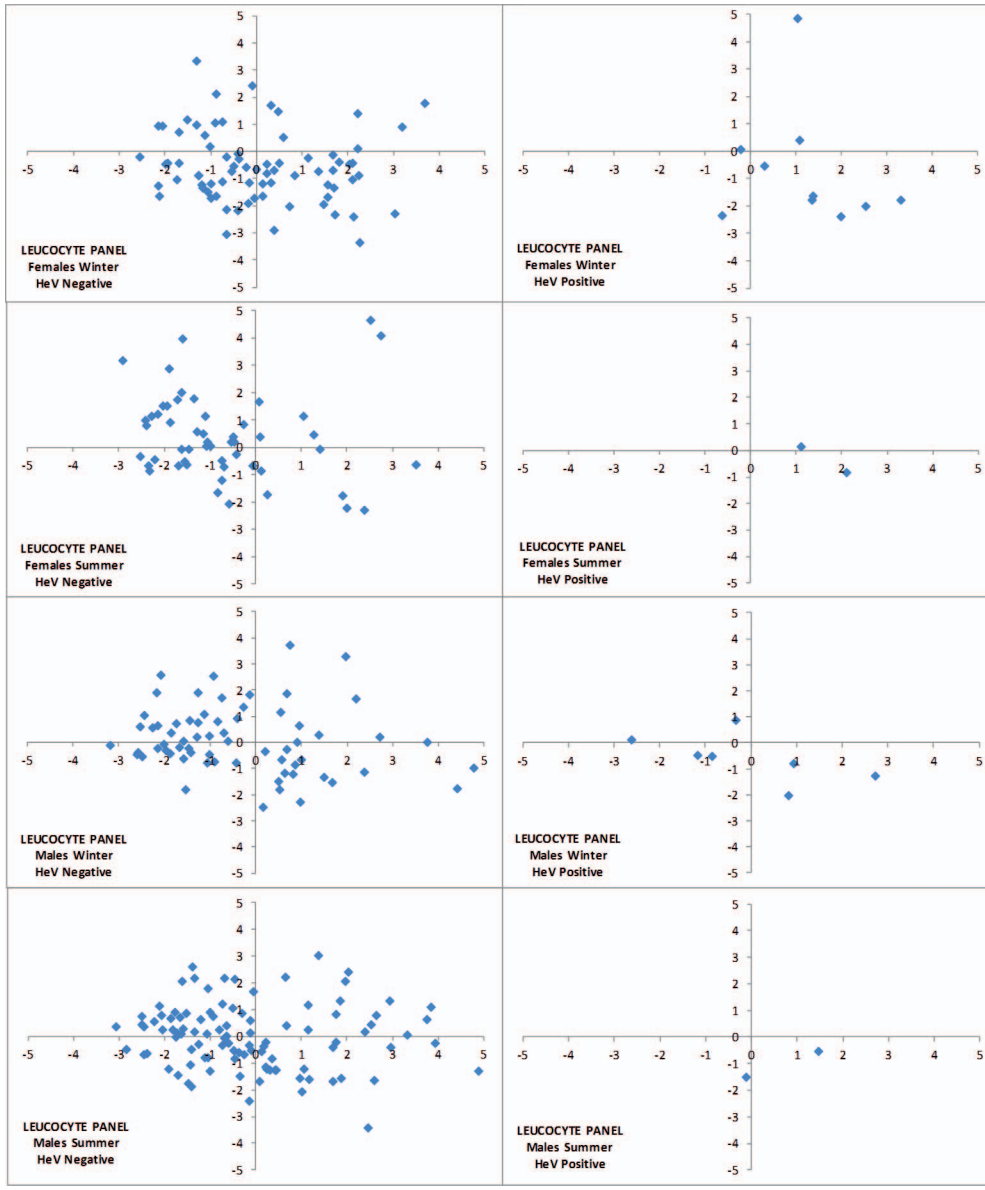

FIGURE 2. Principal component analysis comparing the leukocyte biomarker panel of Hendra virus (HeV)–positive and HeV–negative cohorts for gender and season of black flying foxes (*Pteropus alecto*) sampled in Australia, 2013–14.

er neutrophil percentage in the Hendra virus–positive cohort. However, the latter findings should be interpreted with care in the absence of a statistically significant increase in lymphocyte numbers and the absence of leukocyte cellular morphologic changes indicative of an inflammatory process. Heard and Huft (1998) reported an effect of short-term physical restraint on lymphocyte and neutrophil numbers, but the random nature of our postcapture sampling precludes this explanation, as any such effect would equally affect Hendra virus–positive and virus–negative bats.

McMichael et al. (2015) reported a statistically significant association between lower

hemoglobin, hematocrit and total red cell counts, and decreasing body condition, and similar findings have been reported in other wildlife species (Ruykys et al. 2012). Both the PCA and GLM demonstrated no significant association between erythrocyte biomarkers and Hendra virus infection status in this study. Thus, albeit indirectly, the absence of an association between Hendra virus status and erythrocyte biomarkers argues against an association between Hendra virus status and body condition.

Plasma biochemistry

The plasma biochemistry findings also do not support an association between Hendra virus status and nutritional status. McMichael et al. (2015) reported that animals in poor body condition had lower plasma total protein, albumin, and globulin levels than those in good body condition. Similar findings have been reported in other *Pteropus* species (McLaughlin et al. 2007; Hossain et al. 2013) and plausibly suggest inadequate proteinaceous nutrition. We found no association between Hendra virus RNA status and plasma glucose, total protein, or albumin. In addition, Hendra virus RNA was not associated with elevated urea, creatinine, bilirubin, or creatinine kinase, biomarkers that might indicate catabolism of muscle protein during a state of poor nutrition (Clarke et al. 2013). Further, there was no significant correlation with the majority of individual enzymes that indicate metabolic dysfunction in a wide range of tissues, including the liver, heart, brain, skeletal muscle, and erythrocytes. Finally, the PCA demonstrated no differences due to Hendra virus infection status for the nutritional and metabolic biomarker panels. We acknowledge that the use of hematologic and biochemical data to assess nutritional status is an indirect measure of whether food reserves are meeting physiologic requirements but suggest the approach is a robust and practical alternative to complex field studies exploring seasonal foraging behavior, dietary analysis, and the energetic demands of *Pteropus* species.

The statistically significant, but small, magnitude decrease in plasma triglycerides associated with Hendra virus infection warns against overinterpretation of the biologic significance of the finding. Although McMichael et al. (2015) found that animals experiencing physiologic demand, such as mating and lactation, and those in poor body condition, had lower plasma triglyceride levels, the lack of corroborative support from other biomarkers used to assess long-term planes of nutritional status (such as albumin and urea) does not indicate a nutritional affect (Srivastava and Krishna 2008). It is possible that the association reflects the mobilization and depletion of fatty acids due to the ecologic stressor of lower temperatures during the winter, when Hendra virus is more prevalent in the study population (Field et al. 2015) yet not temporally associated with mating or lactation.

As with plasma triglycerides, although statistically significant, the modest magnitude of the increase in plasma alkaline phosphatase associated with Hendra virus infection again argues against overinterpretation. There was no concomitant increase in levels of gamma-glutamyl transferase, alanine aminotransferase, or aspartate aminotransferase effectively ruling out hepatic disease. McMichael et al. (2015) reported a correlation between increased plasma alkaline phosphatase levels and juvenile and subadult black flying foxes, but this study includes only adult animals. Clinically, plasma alkaline phosphatase increase could reflect enzyme variability due to bone damage and repair in adult animals or differing physiologic states associated with increased levels of the enzyme, such as steroid-induced isoenzyme. The latter warrants further investigation beyond the scope of this study.

Urinalysis

The PCA of the urinary biomarker panel demonstrated no difference between the Hendra virus–positive and virus–negative cohorts. However, GLM found a significant association between Hendra virus infection

and higher urinary protein levels and a nonsignificant trend toward higher pH. The magnitude of the differences was small and should again be interpreted with caution; however, proteinuria has been reported in localized viral infection in the urinary tract (Kruger et al. 2011), and Goldspink et al. (2015) suggested the kidney as a site of Hendra virus localization. Although there is a recognized relationship between USG and protein (with higher protein expected in more concentrated urine samples), the difference in urine protein content between the Hendra virus–positive and virus–negative cohorts was independent of any difference in USG. Similarly, although very strongly acidic or alkaline urine can affect urine protein estimation when using reagent test strips, the modest difference in pH between the Hendra virus–positive and virus–negative cohorts argues against any such effect here.

We sought to measure the impact of Hendra virus infection on relevant physiologic biomarkers in the black flying fox. We employed a suite of physiologic biomarkers routinely used in human and veterinary medicine to assess clinical health and disease status. We found no evidence that Hendra virus infection causes clinical disease in black flying foxes, supporting the contention that virus and host have a mature and mutually advantageous relationship and consistent with black flying foxes being a primary reservoir host. Further, we found no association between Hendra virus infection and biomarkers that was consistent with nutritional stress, extreme metabolic demand, or reproductive stress. While acknowledging the temporal and spatial limitations of our study, we suggest our findings highlight the need for critical evaluation of putative risk factors for Hendra virus infection in flying foxes. Our findings do not preclude such associations, but they demonstrate that such associations are not necessarily a feature of infection. Conversely, identified associations between several biomarkers and Hendra virus infection may partly elucidate the physiologic relationships of Hendra virus infection dynamics and provide insights for future epidemiologic studies of Hendra virus and related viruses in the *Pteropus* species.

ACKNOWLEDGMENTS

We acknowledge core project funding from the National Hendra Virus Research Program supported by the Queensland, New South Wales and Australian governments. Doctoral scholarship support for L.M. was provided by the (then) Australian Biosecurity Cooperative Research Centre for Emerging Infectious Diseases (now the New Agency Name) and by the Australian Postgraduate Award program through the University of Queensland. We particularly thank colleagues Lauren Goldspink, Miranda Vidgen, and Debra Melville at the (then) Queensland Centre for Emerging Infectious Diseases (now New Agency Name), Queensland Department of Agriculture regional field officers Perry Jones and Paul Willett at Boonah, Scenic Rim Regional Council environmental officer Andrew McLaughlin, and Boonah Police Station officers for generous and sustained field laboratory and logistic support.

SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://dx.doi.org/10.7589/2016-05-100.

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Submitted for publication 10 May 2016.
Accepted 19 May 2016.