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# HEMATOLOGICAL AND SERUM BIOCHEMICAL REFERENCE VALUES AND COHORT ANALYSIS IN THE GILBERT'S POTOROO (*POTOROUS GILBERTII*)

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*Abstract:* Hematology and serum biochemistry blood values are tabulated for Australia's most critically endangered mammal, the Gilbert's potoroo (*Potorous gilbertii*). Significant differences were found between origin (captive or wild individuals) and age (subadult or adult) of animals. Sex, and presence or absence of *Treponema* infection, had minimal significance on blood values. Typical cell morphology is discussed, and hemoparasite examination identified *Theileria* spp. and *Breinlia* spp. Eighty samples were collected from a population of only 35 individuals, reflective of a population census rather than of a study reliant on statistical extrapolation. These reference ranges and findings will assist in the ongoing health management of this critically endangered species.

*Key words:* hematology, biochemistry, marsupial, Gilbert's potoroo, *Potorous gilbertii*.

## INTRODUCTION

The Gilbert's potoroo (*Potorous gilbertii*) is a small marsupial endemic to the Two Peoples Bay Nature Reserve in the southwest of Western Australia. The Gilbert's potoroo was presumed to be extinct, as there had been no sightings reported after 1870. However, in 1994, a small population was rediscovered at Mount Gardner in the Two Peoples Bay Nature Reserve near Albany. This remnant population is restricted to an 1,800-ha region of heath land within the reserve. An interim recovery plan was prepared for the species, immediately after its rediscovery, recommending that a captive breeding program be established as part of a comprehensive recovery program to insure against the catastrophic loss of the wild population (e.g., through wildfire) and to breed individuals for translocation in order to establish new populations.[8] Eight animals were removed from the wild to establish the founder group for the captive breeding program. According to IUCN criteria,[13] the Gilbert's potoroo is classified as critically endangered.[8] The current population estimate of the Gilbert's potoroo is 35 individuals (Friend, pers. comm., January 2008).

A collaborative health and disease study of the Gilbert's potoroo was undertaken involving the Department of Environment and Conservation (DEC), the Department of Food and Agriculture (DAFWA), and the Perth Zoo. Eighty blood samples were obtained from 35 Gilbert's potoroos over a three-year period. Blood was collected to establish reference intervals, to identify effects of disease, and to explore factors which may significantly alter hematologic and serum biochemical values in the Gilbert's potoroo.

## MATERIALS AND METHODS

Wild potoroos were trapped three times per year (March, June, and November), by DEC staff, to monitor the population in accordance with the Gilbert's Potoroo Recovery Plan ratified by the Gilbert's Potoroo Recovery Team. Eighty samples were collected from a population of only 35 individuals, comprising 20 males and 15 females, reflective of a population census rather than of a study reliant on statistical extrapolation. Of the 35 individuals, 12 were subadults and 23 were classed as adults, based on tooth eruption and weight. Nine captive and 26 wild individuals were sampled. Over three years, six trapping sessions were conducted. Many of the captive individuals were retrapped over the six trapping sessions. However, there was a minimum of three months between samplings. Therefore, the middle data point for each potoroo was used to calculate the reference interval for that individual, thereby justifying the use of repeated samples in the

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population data. Overall, the number of blood samples collected differed from the number of individuals trapped. If a female had pouch young thought to be of 'critical size' in relation to vulnerability and survivability, then general anesthesia for blood-sample collection was not undertaken. Although this may bias the population, only four females belonged to this category. It was thought that the risk of anesthesia for blood-sample collection far outweighed the study value for this cohort of Australia's most critically endangered mammal.

On Mount Gardner, Sheffield cage traps were set the afternoon prior to trapping and were checked at dawn. When a potoroo was found, it was transferred to a black cotton bag and a total weight was obtained with the use of spring scales. Captive Gilbert's potoroos were captured using a shade-cloth run and net on a fortnightly basis to enable regular weighing and physical examination.

The potoroo's nose was exteriorized from the black cotton bag and the animal was induced with isoflurane (Veterinary Companies of Australia, Kings Park, NSW 2148, Australia), delivered via face mask at 3.5% isoflurane, with an oxygen flow rate of 2.5 L/min. Five minutes postinduction, most potoroos were able to be maintained on 1.5% isoflurane and 2 L/min of oxygen.

The potoroos were scored based on the amount of fat overlying their thoraco-lumbar spine and tail base. Body condition was graded from one to five, one being emaciated and five being obese. A patch of hair overlying the left femoral triangle was clipped with WAHL® clippers (Wahl Clipper Corporation and Unity Agencies, Victoria 3180, Australia) and the skin prepared with a combined 50:50 chlorhexidine gluconate and 70% ethanol mixture. A 23-ga needle and a 3-ml syringe were used to obtain a blood sample from the femoral vein. Up to 0.5% of body weight in a healthy individual was recommended by P. Clark (pers. comm., May 2005). The blood obtained was divided for different test procedures. A blood smear was made via the spreader slide technique for differential leukocyte and platelet counts, assessment of platelet, white blood cell (WBC) and red blood cell (RBC) morphology, and hemoparasite examination. Approximately 0.5 ml of blood was preserved in a 1.5-ml ethylene diamine tetraacetic acid (EDTA) tube and sent with the blood smear to the Murdoch University Department of Veterinary Clinical Pathology (Australia) for a complete blood cell count. An additional 3.5 ml of blood was collected and placed into a tube with no additives and was later centrifuged to obtain serum. Of this total sample, 0.5 ml of serum was submitted for biochemical analysis and 0.5 ml of serum was submitted for the direct agglutination test (DAT) and the modified agglutination test (MAT) for toxoplasmosis at Murdoch University. An additional 0.5 ml of serum was submitted for the latex cryptococcal antigen test at the University of Sydney. Moreover, 0.5 ml of serum was submitted for *Treponema pallidum* particle agglutination (TPPA), enzyme immunoassay (EIA), and the nonspecific rapid plasmid reagin (RPR) test for detection of *Treponema*. These samples were sent via overnight courier, on ice, to Pathwest, Nedlands, Western Australia. Any excess serum collected was frozen at $-20^{\circ}C$ for two days and then transferred to a $-80^{\circ}C$ freezer for future use. Urine was collected mid-stream free-catch for urinalysis and forwarded via overnight courier, on ice, to Murdoch University.

Complete blood cell counts were performed on whole blood using EDTA anticoagulant on an Advia 120 hematology analyzer (Bayer, Tarrytown, New York 10591, USA). RBC count, WBC count, hemoglobin (Hb), packed cell volume (PCV), mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC) were measured. Blood films were stained with Wright Giemsa stains, and differential leukocyte counts, RBC and WBC morphology, and hemoparasite examination were conducted under light microscopy. Fibrinogen concentration was determined using the heat precipitation method. Alkaline phosphatase (ALP), aspartate amino transferase (AST), alanine transaminase (ALT), creatinine kinase (CK), total bilirubin, urea, creatinine, cholesterol, calcium, phosphorous, glucose, albumin, globulin, and total serum protein were analyzed using a Randox RX Daytona analyzer (Randox Laboratories Ltd., Belfast BT3 9HA, Ireland). Total protein was measured using the Daytona Biuret Reagent method, and urea was measured using the Daytona Enzymatic Kinetic Method. Urinalysis was performed using Multistix® Reagent strips (Bayer, Pymble, 2073 Australia) and urine specific gravity measured through the use of a refractometer.

Data was recorded using an Excel spreadsheet (Microsoft Corporation, North Ryde, NSW 2113, Australia). A determination of health was made based on physical examination findings, recovery postprocedure, and the absence of any history of current or chronic illness. Four

individuals were deemed unhealthy and excluded from the study. Captive Gilbert's potoroo (GP) F66, an azotemic, aged potoroo, had elevated blood urea nitrogen (BUN), 26.6 mmol/L (mean: 5.946 SD: 1.5036), creatinine 334 μmol/L (mean: 49.52 SD 13.043), and clinical evidence of poluria and polydipsia. These findings were consistent with a diagnosis of chronic renal disease, which was later confirmed via histopathology following this animal's natural death. Captive GP (F57) had a prolonged and stressful capture in November 2005. This female had a resultant increased CK activity of 20,000 U/L (mean 1074.4, SD 924.29) and AST 1161 U/L (mean 84.64, SD 43.37), indicative of acute muscle damage. No increase in liver enzymes was present. Wild female GP (F61) had an eosinophilia of 15% (mean 2.73, SD 1.42), and a total count of $0.59 \times 10^9$/L (mean 0.08, SD 0.06), in March 2007; the etiology is unknown. Other hematology results were unremarkable, and a minimal ectoparasite burden was present. Parasitism is a common cause of eosinophilia.[25] Hence, this value was classed as an outlier, although the possibility of laboratory error could not be ruled out. The final individual was an aged, wild GP (M55) who was in poor body condition (condition score 1.5), had pale mucous membranes, a prolonged recovery from anesthesia, and hematologic evidence of anemia in July 2005 with an HCT: 0.16 L/L (mean 0.38, SD 0.03); RBC: $2.53 \times 10^{12}$/L (mean 6.20, SD 0.72); and Hb 26 g/L (mean 125.82, SD 11.91).

Statistical analysis was performed using SPSS® (Version 15 for Windows, SPSS.com, Chicago, Illinois 60630, USA) to create reference intervals for the population of 35 and also to present the upper and lower 95% confidence interval, mean, and standard error. These confidence intervals were generated in analysis of variance (ANOVA) and *t*-test statistical programs and did not necessarily represent the data in the same manner as the reference values. Therefore, minimum and maximum ranges, with outliers excluded of data, have also been presented so clinicians can review the data collected. (K. Harr, pers. comm., November 2008). Statistical analysis, including a comparison of cohorts, included age (subadults compared to adults), origin (captive compared to wild), presence of *Treponema* infection, and sex (males compared to females) via a one-way ANOVA, was also performed using SPSS. Statistical significance was assessed at a level of $P < 0.05$.

*Treponema* infection in male Gilbert's potoroos presents as a moderate–severe balanoposthitis with an associated green discharge. *Treponema* infection was diagnosed via a number of techniques. In males, the penis was extruded and any discharge was described. A dry wire swab (Mini-tip swabs, COPAN, Murrieta, California 92562, USA) was used to swab the prepuce for the detection of *Treponema* infection via a polymerase chain reaction (PCR) assay (Buller and Vaughan, DAFWA, Western Australia). These results were also cross-referenced with dark-field microsopy results for spirochetes. The swab was stored frozen at −20°C degrees prior to transport via overnight courier to DAFWA. The prepuce was then swabbed with a moistened Amie's transport medium swab (Transwab®, Medical Wire & Equipment, Corsham, Wiltshire SN13 9RT, United Kingdom), which was first used to make a wet-preparation smear for the detection of spirochetes under dark-field microscopy and then forwarded to the DAFWA for microbiologic analysis and culture. In females, the vagina was swabbed, and the presence of any discharge, and its characteristics, were recorded. A dry wire swab (Mini-tip swabs®, COPAN, Murrieta, California 92562, USA) was used for the detection of *Treponema* infection via PCR assay. The vagina was then swabbed with a moistened Amie's transport medium swab (Transwab®, Medical Wire & Equipment, Corsham, Wiltshire SN13 9RT, United Kingdom), which was used to make a wet-preparation smear for the detection of spirochetes under dark-field microscopy, prior to being shipped overnight to DAFWA for microbiologic analysis and culture. Definitive diagnosis of *Treponema* infection was based on presence of discharge, positive PCR, and positive dark-field microscopy for spirochetes. Given the severity of associated clinical signs, it was decided to examine hematologic and biochemical differences between infected and noninfected individuals.

## RESULTS

Despite careful collection, some blood samples were slightly hemolyzed. Hemolysis can alter the results of serum analytes.[1] However, the values obtained from the hemolyzed samples seemed comparable to unaffected samples and were included in the results. The results of measured hematologic and biochemical analytes are found in Table 1.

### Blood smear examination

Examination of the blood film revealed cells similar to other mammals. The erythrocytes were

**Table 1.** Hematologic and biochemical values for Gilbert's potoroo.

| Analytes ($n$ = 35) | Abbreviation | Mean | Standard error | Upper 95% CI for mean | Lower 95% CI for mean | Standard deviation | Minimum value | Maximum value |
|---|---|---|---|---|---|---|---|---|
| Hemoglobin (g/L) | Hb | 125.82 | 3.59 | 133.82 | 117.82 | 11.91 | 94 | 157 |
| Hematocrit (L/L) | HCT | 0.38 | 0.11 | 0.41 | 0.36 | 0.04 | 0.29 | 0.49 |
| Packed cell volume (L/L) | PCV | 0.36 | 0.11 | 0.39 | 0.34 | 0.037 | 0.28 | 0.46 |
| Red blood cell count | RBC | 6.20 | 0.22 | 6.69 | 5.72 | 0.72 | 2.53 | 8.24 |
| Mean cell Hb concentration | MCHC | 330.27 | 3.20 | 337.41 | 323.13 | 10.63 | 303 | 354 |
| Mean corpuscular Hb | MCH | 20.34 | 0.32 | 21.05 | 19.62 | 1.06 | 18.6 | 21.8 |
| Mean corpuscular volume | MCV | 61.6 | 0.96 | 63.73 | 59.47 | 3.175 | 56.10 | 61.72 |
| White blood cell count | WBC | 3.12 | 0.31 | 3.81 | 2.43 | 1.03 | 0.60 | 9.40 |
| Neutrophils (%) | Neut | 33.27 | 4.26 | 41.76 | 22.79 | 14.12 | 2 | 72 |
| Neutrophils ($\times 10^9$/L) | Neut | 0.93 | 0.11 | 1.172 | 0.69 | 0.35 | 0.16 | 2.23 |
| Lymphocytes (%) | Lymph | 58.36 | 4.96 | 69.41 | 47.32 | 16.44 | 26 | 90 |
| Lymphocytes ($\times 10^9$/L) | Lymph | 1.89 | 0.31 | 2.58 | 1.22 | 1.01 | 0.23 | 7.13 |
| Monocytes (%) | Mono | 1.68 | 0.32 | 0.24 | 0.97 | 1.05 | 1 | 10 |
| Monocytes ($\times 10^9$/L) | Mono | 0.49 | 0.01 | 0.07 | 0.03 | 0.03 | 0.01 | 0.20 |
| Eosinophils (%) | Eosin | 2.73 | 0.43 | 3.68 | 1.77 | 1.42 | 0 | 15 |
| Eosinophils ($\times 10^9$/L) | Eosin | 0.08 | 0.02 | 0.13 | 0.04 | 0.06 | 0.03 | 0.59 |
| Basophils (%) | Baso | 1.36 | 0.20 | 1.82 | 0.91 | 0.67 | 1 | 3 |
| Basophils ($\times 10^9$/L) | Baso | 0.43 | 0.01 | 0.06 | 0.023 | 0.03 | 0.02 | 0.11 |
| Platelet count ($\times 10^9$/L) | | 345.45 | 39.55 | 433.59 | 257.32 | 131.19 | 71 | 578 |
| Total protein (g/L) | TP | 61.09 | 0.59 | 62.42 | 59.77 | 1.97 | 56 | 75 |
| Fibrinogen (g/L) | | 1.45 | 0.21 | 1.91 | 0.98 | 0.69 | 1 | 3 |
| Creatine kinase (U/L) | CK | 1074.14 | 260.39 | 1636.68 | 511.6 | 924.29 | 157 | 8978 |
| Aspartate transferase (U/L) | AST | 84.64 | 11.59 | 109.68 | 59.6 | 43.36 | 44 | 161 |
| Alanine aminotransferase (U/L) | ALT | 76.93 | 7.19 | 92.48 | 61.38 | 26.93 | 33 | 298 |
| Alkaline phosphatase (U/L) | ALP | 318.64 | 39.19 | 403.32 | 233.96 | 146.66 | 88 | 1327 |
| Total bilirubin (mmol/L) | BILI | 4.69 | 0.91 | 6.66 | 2.72 | 3.41 | 0 | 12 |
| Urea (mmol/L) | BUN | 5.95 | 0.41 | 6.81 | 5.08 | 1.50 | 2.10 | 28.60 |
| Creatinine (mmol/L) | CREAT | 49.52 | 3.48 | 57.05 | 41.99 | 13.04 | 33 | 334 |
| Glucose (mmol/L) | GLU | 9.21 | 0.61 | 10.54 | 7.87 | 2.30 | 5.80 | 20.10 |
| Cholesterol (mmol/L) | CHOL | 3.75 | 0.22 | 4.23 | 3.272 | 0.83 | 26 | 6.50 |
| S protein (g/L) | SP | 56.61 | 1.67 | 60.22 | 53.01 | 6.24 | 45.30 | 73.10 |
| Albumin (g/L) | ALB | 33.24 | 0.59 | 34.51 | 31.96 | 2.21 | 28 | 43 |
| Globulin (g/L) | GLOB | 23.36 | 1.74 | 27.13 | 19.60 | 6.51 | 13.70 | 36.10 |
| A:G ratio | A:G | 1.54 | 0.14 | 1.84 | 1.25 | 0.51 | 0.94 | 2.75 |
| Calcium (mmol/L) | Ca | 2.03 | 0.06 | 2.159 | 1.89 | 0.23 | 1.30 | 2.62 |
| Phosphorus (mmol/L) | P | 2.84 | 0.25 | 3.38 | 2.30 | 0.94 | 1 | 4.5 |

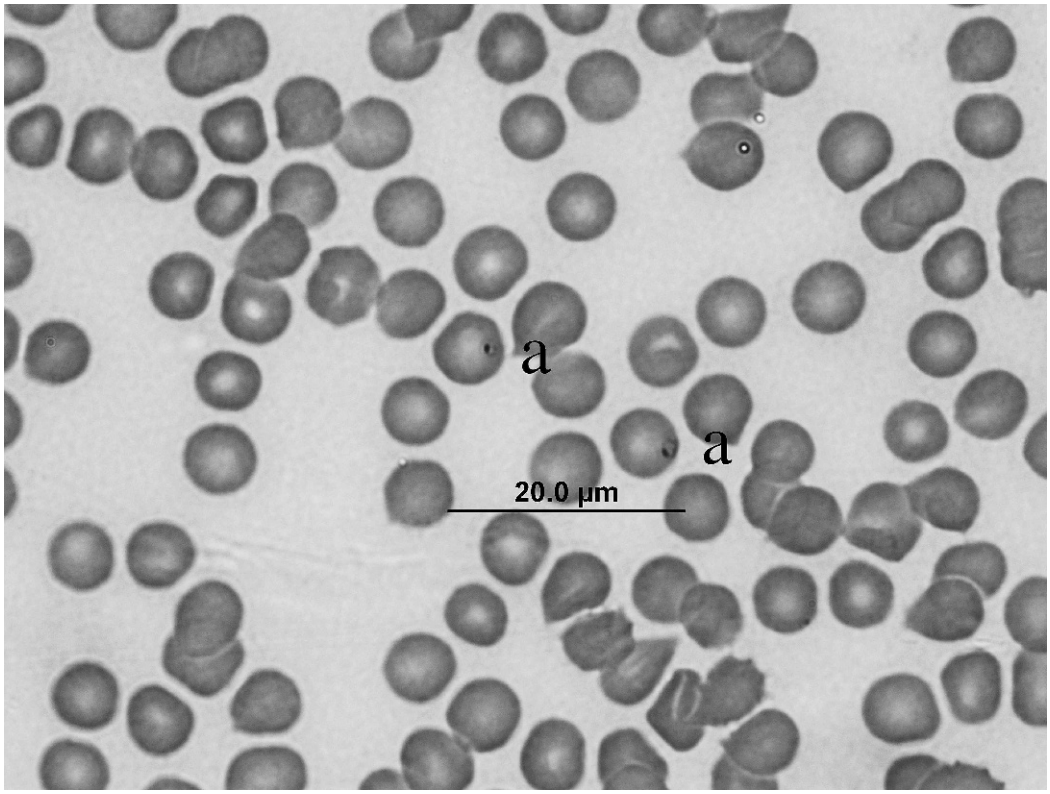


**Figure 1.** Peripheral blood smear of a Gilbert's potoroo showing the presence of **a.** intra-erythrocytic *Theileria*. **b.** The piroplasms were round, ovoid, or pear shaped with 1–2 parasites present per cell. Wright and Giemsa stain ×100.

nonnucleated eosinophilic bioconcave discs with mild to moderate central pallor. Erythrocytes stained pale pink in Wright-stained preparations. Occasional Howell-Jolly bodies and mild anisocytosis were also evident in healthy individuals. Neutrophils had between three to six nuclear lobes, with chromatin clumping and a colorless cytoplasm. Lymphocytes were typically small- to medium-sized cells and had a round nucleus, chromatin clumping, and basophilic cytoplasm. Monocytes had an irregularly shaped nucleus and a basophilic cytoplasm, with occasional vacuolation. Eosinophils, when present, had between two to four lobes, with chromatin clumping, and had diffuse eosinophilic granules present throughout the cytoplasm. Basophils had dark basophilic granules diffusely present throughout the cytoplasm. No alterations in leukocyte morphology were evident when comparing blood films of those with *Treponema* infection to those lacking *Treponema* infection.

Red-cell hemoparasites were seen in three captive potoroos, GP (M46), (F57), and (F93), in June 2006, November 2006, and March 2007, respectively. There was a high degree of parasitemia, with at least two red cells infected per high-power field. Morphologically, the inclusions were pleomorphic, round, or irregularly shaped intra-erythrocytic parasites, approximately 1–2 µm in length, and were morphologically consistent with *Theileria* spp. (Fig. 1). They were distinguished by their size, the spherical ring, their presence in lymphocytes, and the smaller amount of nuclear material compared to the piroplasm, *Babesia*. No evidence of anemia, red cell morphology changes, or inflammation was seen.

In June 2006, wild GP (M68) and GP (M116) had a similar high level of parasitemia with *Theileria* spp. In November 2006, high parasitemia with *Theileria* spp. was again noted with a corresponding microfilaria infection in both individuals (Fig. 2). This hemoparasite was morphologically consistent with *Breinlia* spp., due to its long, unsheathed, tapering tail.[5] Again, there was no evidence of anemia, red-cell morphology changes, or inflammation in either individual.

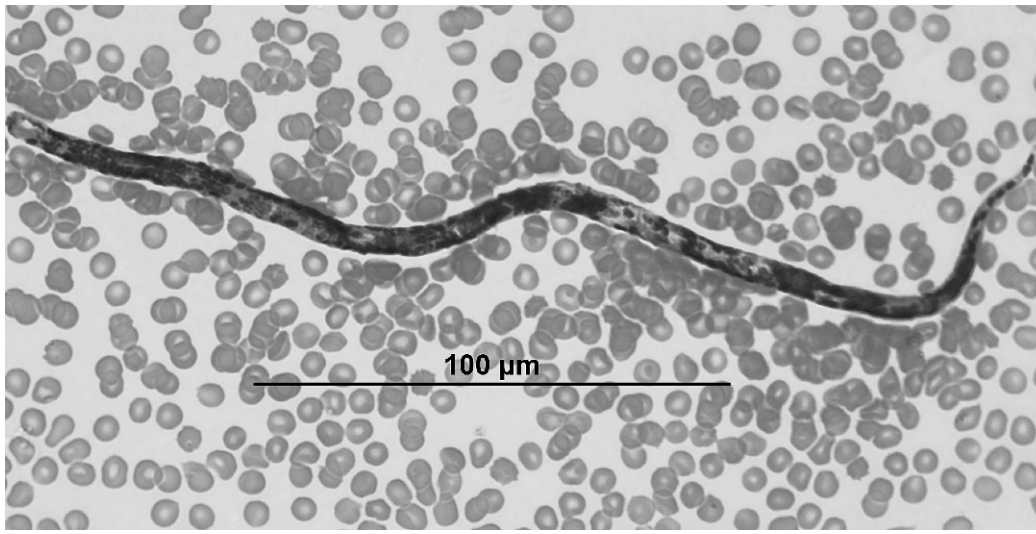


**Figure 2.** Microfilaroid in a peripheral blood smear of a Gilbert's potoroo. Wright and Giemsa stain ×40.

### Hematologic and biochemical evaluation

Tests of normality conducted with SPSS software (Chicago, Illinois 60630, USA) included the Kolmogorov-Smirnov statistic, a histogram, detrended normal Q-Q plot, and a boxplot. Apart from monocytes (%), basophils (%), basophils ($\times 10^9$), and fibrinogen, all hematologic analytes were found to be normally distributed. The basophil and monocyte distribution was positively skewed, with the majority of values clustered to the left of the histogram reflecting low values. Fibrinogen was similarly positively skewed to the left, reflecting a majority of low values; however, the kurtosis value reflected a lack of values in the center of the distribution, leading to asymmetry. Serum biochemistry results of normality indicated a normal distribution of analytes other than CK, AST, creatinine, and Ca. For all of these analytes, there were a low number of values in the center of the distribution, with a positive skew to the left, indicating a trend towards low values. These four analytes also had one high outlier, further skewing the distribution to the left, minimizing kurtosis, and resulting in asymmetry.

Age, origin, sex, and *Treponema* infection, in relation to hematologic and biochemical parameters, were then examined. Age effects were evident in subadults as compared to adults. Subadults returned significantly greater values for PCV ($P = 0.000$), WBC ($P = 0.000$), lymphocytes ($P = 0.000$), basophils ($P = 0.017$), total protein ($P = 0.033$), fibrinogen ($P = 0.045$), ALP ($P = 0.000$), glucose ($P = 0.05$), and calcium ($P = 0.002$) (Table 2). Origin effects were evident in captive compared to wild individuals. Captive individuals had significantly greater values for Hb ($P = 0.000$), PCV ($P = 0.000$), RBC ($P = 0.000$), ALP ($P = 0.03$), cholesterol ($P = 0.000$), and albumin ($P = 0.000$). Wild individuals had significantly greater values for CK ($P = 0.001$), AST ($P = 0.03$), total bilirubin ($P = 0.004$), urea ($P = 0.000$), creatinine ($P = 0.000$), globulin ($P = 0.002$), and phosphorus ($P = 0.000$) (Table 3). Gender effects were minimal; males had significantly greater values only for fibrinogen ($P = 0.029$), creatinine ($P = 0.042$), and cholesterol ($P = 0.009$). When comparing the *Treponema*-infected population to the non-*Treponema*-infected population, minimal differences were seen in the following analytes: The *Treponema*-infected individuals had lower cholesterol ($P = 0.041$) and ALP ($P = 0.000$), with higher serum protein ($P = 0.032$) results. None of these observations were thought to be clinically significant, and all fell within the reference interval for the total population. No serologic response was detected to any of the human-based *Treponema* tests.

## DISCUSSION

### Blood smear examination

Blood smears were examined as Clark[7] had previously reported that the recognition of clinical inflammatory disorders in macropodids,

**Table 2.** Statistically significant effects of age on hematologic and biochemical values for Gilbert's potoroo.

| Analytes ($n$ = 35) | Subadult (SA) ($n$ = 12) or adult (A) ($n$ = 23) | Mean | Standard error | Upper 95% CI[a] for mean | Lower 95% CI for mean | Standard deviation | Minimum value | Maximum value |
|---|---|---|---|---|---|---|---|---|
| Packed cell volume (L/L) | SA | 0.37 | 0.01 | 0.45 | 0.29 | 0.30 | 0.33 | 0.46 |
| | A | 0.36 | 0.01 | 0.39 | 0.32 | 0.04 | 0.28 | 0.44 |
| White blood cell count | SA | 4.33 | 0.67 | 7.23 | 1.43 | 1.17 | 2.00 | 9.40 |
| | A | 2.66 | 0.18 | 3.08 | 2.24 | 0.51 | 0.60 | 5.10 |
| Lymphocytes (%) | SA | 75 | 2 | 83.61 | 66.39 | 3.46 | 45 | 90 |
| | A | 52.13 | 5.24 | 64.51 | 39.74 | 14.81 | 28 | 88 |
| Lymphocytes ($\times 10^9$/L) | SA | 3.22 | 0.42 | 5.01 | 1.42 | 0.72 | 0.95 | 7.13 |
| | A | 1.41 | 0.19 | 0.90 | 1.86 | 0.94 | 0.23 | 4.08 |
| Basophils (%) | SA | 1.67 | 0.33 | 3.10 | 0.23 | 0.57 | 1 | 2 |
| | A | 1.25 | 0.25 | 1.84 | 0.66 | 0.71 | 1 | 3 |
| Basophils ($\times 10^9$/L) | SA | 0.07 | 0.02 | 0.174 | 0.00 | 0.04 | 0.03 | 0.11 |
| | A | 0.03 | 0.01 | 0.04 | 0.02 | 0.01 | 0.02 | 0.07 |
| Total Protein (g/L) | SA | 61.33 | 0.88 | 65.13 | 57.54 | 1.53 | 59.00 | 65 |
| | A | 61 | 0.78 | 62.84 | 59.16 | 2.20 | 50.00 | 75 |
| Fibrinogen (g/L) | SA | 1.67 | 0.67 | 4.54 | 1.2 | 1.15 | 1.00 | 3.00 |
| | A | 1.36 | 0.19 | 1.8 | 0.92 | 0.53 | 0.90 | 3.00 |
| Alkaline phosphatase (U/L) | SA | 467.25 | 90.19 | 754.28 | 180.22 | 180.38 | 54.00 | 152 |
| | A | 259.2 | 25.46 | 316.8 | 201.6 | 80.52 | 88.00 | 481 |
| Glucose (mmol/L) | SA | 9.87 | 1.40 | 14.34 | 5.40 | 2.81 | 6.40 | 20.10 |
| | A | 8.94 | 0.69 | 10.50 | 7.37 | 2.18 | 6.00 | 17.50 |
| Calcium (mmol/L) | SA | 2.11 | 0.18 | 2.69 | 1.53 | 0.37 | 1.79 | 2.62 |
| | A | 1.99 | 0.05 | 2.11 | 1.88 | 0.16 | 1.30 | 2.32 |

[a] CI, confidence interval.

using only cell counts, was difficult. It was proposed that morphologic changes often provide a more-consistent indication of inflammation. The erythrocyte and leukocyte morphology was similar to that described by Clark[5] and by Moore and Gillespie[19] in the Gilbert's and long-nosed potoroo, respectively. The piroplasm (*Theileria* spp.) previously reported in Gilbert's potoroos[15] was found in three captive potoroos over three sequential trapping sessions spanning 10 mo. Two wild males were also found to have a high parasitemia, with at least two red cells infected per high-power field. One of these individuals remained persistently infected for the following 5 mo. This individual was not retrapped in March 2007 and, as such, no further disease screening was undertaken.

In the captive population, the presence of *Theileria* spp. was not associated with regenerative anemia. However, the wild population did have evidence of a regenerative anemia, indicated by reduced PCV, Hb, RBC number, and polychromasia of erythrocytes; and yet, interestingly, this population (other than two individuals) lacked *Theileria* spp. This is consistent with the findings of Lee,[15] which did not indicate pathologic consequences from the presence of *Theileria* spp. in the Gilbert's potoroo. This observation is similar to the minimal host effect of *Theileria ornithorhynchi* found commonly in the platypus[17] and suggests that certain species of piroplasms and mammals may have evolved together, with minimal associated pathologic effect.[15] It is possible that ticks, which are commonly found on Gilbert's potoroo, are responsible for the transmission of the organism; however, this requires further investigation.

One wild male individual, in suboptimal body condition, had evidence of a low level of infection, with a single microfilaria found on peripheral blood smear. This hemoparasite was identified to be a *Breinlia* spp. No corresponding increased concentration of WBCs was noted. Given the low levels of microfilaria found, and the lack of associated eosinophilia, it is difficult to assign significance to the presence of microfliaria in this individual. However, further evaluation of this parasite is warranted. Concentration techniques to enhance detection, thoracic radiographs, and PCR could also be considered. It is recommended that hemoparasite screening, via blood smears, should be continued as part of

**Table 3.** Statistically significant effects of origin on hematologic and biochemical values for Gilbert's potoroo.

| Analytes ($n$ = 35) | Origin: Wild ($n$ = 26) or captive ($n$ = 9) | Mean | Standard error | Upper 95% CI[a] for mean | Upper 95% CI for mean | Standard deviation | Minimum value | Maximum value |
|---|---|---|---|---|---|---|---|---|
| Hemoglobin (g/L) | Wild | 124 | 5.08 | 136.43 | 111.57 | 13.44 | 94.90 | 141 |
| | Captive | 129 | 4.74 | 144.10 | 113.9 | 9.48 | 115 | 157 |
| Packed cell volume (L/L) | Wild | 0.35 | 0.01 | 0.38 | 0.31 | 0.04 | 0.28 | 0.42 |
| | Captive | 0.39 | 0.01 | 0.41 | 0.37 | 0.01 | 0.33 | 0.44 |
| Red blood cells | Wild | 5.96 | 0.27 | 6.62 | 5.31 | 0.71 | 2.53 | 7.34 |
| | Captive | 6.63 | 0.30 | 7.58 | 5.67 | 0.60 | 5.37 | 7.85 |
| Creatine kinase (U/L) | Wild | 1327 | 332.01 | 2078.06 | 575.94 | 1049.91 | 395 | 8978 |
| | Captive | 442 | 122.97 | 833.37 | 50.63 | 245.95 | 430 | 3694 |
| Aspartate transferase (U/L) | Wild | 88.5 | 16.19 | 125.12 | 51.88 | 51.19 | 51 | 417 |
| | Captive | 75 | 5.54 | 91.99 | 58.01 | 10.67 | 55 | 273 |
| Alkaline phosphatase (U/L) | Wild | 309.9 | 53.35 | 430.59 | 189.21 | 168.71 | 88 | 878 |
| | Captive | 340.5 | 41.6 | 472.92 | 203.08 | 83.22 | 137 | 1327 |
| Total bilirubin (mmol/L) | Wild | 5.6 | 1.01 | 7.89 | 3.31 | 3.20 | 0.90 | 11.90 |
| | Captive | 2.43 | 1.57 | 7.44 | 0.00 | 3.154 | 0.10 | 8.00 |
| Urea (mmol/L) | Wild | 6.64 | 0.35 | 7.45 | 5.87 | 1.09 | 3.5 | 11.40 |
| | Captive | 4.15 | 0.19 | 4.76 | 3.33 | 0.39 | 2.1 | 11.80 |
| Creatinine (mmol/L) | Wild | 49.73 | 4.91 | 60.83 | 36.83 | 15.52 | 33 | 90 |
| | Captive | 49 | 1.87 | 54.95 | 43.05 | 3.74 | 35 | 139 |
| Cholesterol (mmol/L) | Wild | 3.34 | 0.02 | 3.94 | 2.97 | 0.67 | 2.70 | 4.40 |
| | Captive | 4.49 | 0.37 | 5.69 | 3.29 | 0.75 | 2.70 | 6.52 |
| Albumin (g/L) | Wild | 32.26 | 0.50 | 33.39 | 31.22 | 1.59 | 27.80 | 43 |
| | Captive | 35.67 | 0.79 | 38.19 | 33.16 | 1.58 | 31.20 | 40.10 |
| Globulin (g/L) | Wild | 25.85 | 1.85 | 30.04 | 21.65 | 5.86 | 15.30 | 36.10 |
| | Captive | 17.15 | 1.47 | 21.84 | 12.46 | 2.94 | 13.70 | 27.70 |
| Phosphorus (mmol/L) | Wild | 3.19 | 0.26 | 3.78 | 2.59 | 0.84 | 1.50 | 4.50 |
| | Captive | 1.97 | 0.27 | 2.85 | 1.1 | 0.55 | 1 | 4.20 |

[a] CI, confidence interval.

a health screening for any potoroo that will be translocated, or undergoing health evaluation for diagnostic purposes.

### Hematology and biochemical examination

Numerous blood reference ranges were examined for marsupials.[3,5,11,12,19,21,22,26,27,29,30,31] Many of these studies reported hematologic findings with no biochemistry data, or the methods of biochemical analysis were not reported, making comparison of analytes difficult.

The mean WBC count for the Gilbert's potoroo was $3.118 \times 10^9$/L $\pm$ 1.03. This value is considerably lower than that of the long-nosed potoroo (*Potorous tridactylus*; $8.06 \times 10^9$/L $\pm$ 2.59)[17] and the rufous bettong (*Aepyprymnus rufescens*; $6.49 \times 10^9$/L $\pm$ 0.63; Spencer et al., unpubl. data). These potoroid species were thought to be the most similar, in terms of digestive system, to the Gilbert's potoroo and, thus, serve as a useful comparison.

Like most marsupials, the predominant circulating leukocyte in the Gilbert's potoroo was the lymphocyte.[5] The absolute mean number of lymphocytes ($1.89 \times 10^9$/L) was comparable to other marsupial species, including the mountain brushtail possum (*Trichosurus caninus*; $1.6 \times 10^9$/L),[30] yet was considerably different from wild-allied rock wallabies (*Petrogale assimilis*; $5.22 \times 10^9$/L),[25] the long-nosed potoroo,[18] and the rufous bettong (Spencer et al., unpubl. data). However, it should be highlighted that only relative values were reported for the analogous potoroid species; no absolute values were reported.

A study of 29 morbid members of the Macropodidae revealed that a change in neutrophil concentration was the most consistent indicator of inflammation.[6] However, in Clark's[6] study, the magnitude of change in neutrophil concentration was not dramatic, which led to the conclusion that, when interpreting the leukogram from members of the family Macropdidae, minor deviations from reference ranges should be

considered significant. In this study, an inflammatory challenge, as reported by significant changes in neutrophil concentration and neutrophil morphology,[6] was not present when comparing cohorts including sex, age, origin, or the presence of *Treponema* infection. The four potoroos excluded from this study had no evidence of a neutrophilia, but did have evidence of chronic renal disease, anemia, eosinophilia, and increased muscle enzymes.

Clark[6] reported that a lymphocytosis was also concurrently present in members of the macropodidae, with concurrent alterations in neutrophil characteristics. A relative lymphocytosis was evident in the subadult compared to the adult population ($P = 0.000$). However, this change was thought to be physiologic rather than pathologic. A predominance of immature myeloid cells is typically observed in the blood of marsupial pouch young, with a gradual increase in the number of neutrophils seen over time. This response has also been reported in the eastern quoll (*Dasyurus viverrinus*), common brushtail possum (*Trichosurus vulpecula*), and southern brown bandicoot (*Isoodon obesulus*).[8]

The neutrophil:lymphocyte (N:L) ratio is thought to be a reliable method to detect glucocorticoid-mediated stress in captive animals.[21] In a classic glucocorticoid-mediated stress response, an absolute neutrophilia and lymphopenia is observed, leading to an increased N:L ratio. Although less common, a mild eosinopenia may also be noted.[5] Using the mean leukocyte values, the calculated mean N:L ratio for the total population of the Gilbert's potoroo was 0.49. Given that no statistical significance in leukocyte count was found between wild and captive individuals, it was not thought necessary to compare these two cohorts. Marsupials tend to have N:L ratios of less than 1.[31] Agile wallabies (*Macropus agilis*) have an N:L ratio of 0.5,[26] and quokkas (*Setonix brachyurus*) have a reported N:L ratio of 0.82.[16] The value found in the Gilbert's potoroo, although low, still appears to be within close range to other macropodoids. Absolute N:L ratios were not available for the long-nosed potoroo or the rufous bettong.

Alterations in the N:L ratio have been associated with age, method of restraint, and blood collection.[28] Higher lymphocyte counts are frequently found in younger animals due to establishment of the immune system.[28] Subadult Gilbert's potoroos had a lower N:L ratio (0.29) compared to adults (0.66), as subadults had a statistically higher lymphocyte count ($P = 0.000$). Likewise, in allied rock wallabies, the neutrophil concentration increased with age and resulted in an increase in the N:L ratio from 0.33 at 150 days to 1.0 at maturity.[26] Although the subadult cohort in this study extended to animals between 225 to 250 day postbirth, the N:L ratio still remained relatively low at maturity (0.66), compared to other marsupials. Although the capture technique of the captive compared to wild individuals differed substantially, leukocyte changes were not statistically significant. This is interesting, given that free-living koalas (*Phascolarctos cinereus*) had a persistent neutrophilia up to 6 hr postcapture,[11] and free-living platypus (*Ornithorhyncus anatinus*) had a persistent neutrophilia up to 12 hr postcapture.[30] However, neutrophilia may also be due to an inflammatory process (infectious or noninfectious), or may be indicative of subclinical disease.

Although leukocyte changes were not significant, higher CK and AST activity were evident in the wild compared to the captive population, indicating muscle injury often associated with stressful restraint or capture.[28] Furthermore, the corresponding lack of liver enzyme increase in these wild individuals indicated that the AST increase was likely due to the muscle iso-enzyme rather than to hepatocellular damage. Wild individuals could potentially be trapped for up to 12 hr prior to physical examination and anesthesia, while captive individuals were captured no longer than 1 hr prior to physical examination and anesthesia. Although every attempt was made to minimize the stress of capture for wild individuals (through wrapping traps in hessian bags in order to minimize exposure to the elements and predators), the potential for muscle enzyme elevation with overnight trapping must be considered when comparing these two cohorts.

Subadult Gilbert's potoroos had a higher PCV and total protein concentration compared to adults. Age-related changes, characterized by an increasing Hb, RBC, and hematocrit with age, were reported in the quokka,[31] koala, allied rock-wallaby, and common brushtail possum.[5] The only marsupial species reported to decrease its hematocrit with age is the female mountain brushtail possum.[4]

Wild potoroos had a significantly decreased Hb concentration, PCV, and numbers of RBC. Anemia in macropods has been associated with hemorrhage from nematode infestation,[2] other endoparasites,[25] seasonal nutritional anemia, and undetermined causes. A dominance of strongyloid

nematodes was found in wild Gilbert's potoroos over the course of this study; however, the lack of associated clinical signs and absence of anemia led these endoparasites to be regarded as commensal and of little clinical significance. In Clark's[6] study, anemia was commonly associated with indicators of inflammation, including increased neutrophil concentration, altered neutrophil morphology, or increased fibrinogen concentration. None of these factors were concurrently present in the wild population. It is most likely that the decrease in these parameters, although statistically significant, is not clinically significant. The degree of change in parameters is only mild.

The increased Hb concentration, PCV, and RBC count could be caused by a decrease in plasma volume (relative polycythaemia) or an increase in cells (absolute polycythaemia). However, all captive individuals constantly had water available and very infrequently had gastrointestinal symptoms. The Gilbert's potoroo, unlike some species of macropod, is not adapted for water conservation, whereby significant dehydration to 80% of original body weight can result in only a minimal decrease in plasma volume.[6] Therefore, it seems more likely that the erythron changes represent a redistribution of erythrocytes following catecholamine-mediated splenic contraction[4] in response to capture. In the koala (*P. cinereus*), a similar phenomenon is observed, with the hemoglobin concentration, erythrocyte concentration, and PCV being highest at the time of capture and then decreasing stepwise at 6 hr, 24 hr, and 7 day postcapture.[11] The captive Gilbert's potoroos are handled fortnightly, and these erythron increases could be indicative of repetitive catecholamine-mediated splenic contraction. However, the corresponding lack of leukocyte changes seems to dispel this hypothesis, as a corresponding neutrophilia and lymphocytosis would be expected. Minimal changes in leukocyte numbers have previously been noted (P. Clark, pers. comm., June 2006), and this may be indicative of an atypical glucocorticoid stress response in the Gilbert's potoroo. Unfortunately, a fluoride-containing anticoagulant was not used and, therefore, the blood glucose values cannot be interpreted due to the minimum 20-hr delay in the analysis of blood samples. Further study into glucocorticoid-mediated change is warranted in this species.

Other differences in subadult compared to adult Gilbert's potoroos included significantly greater values for total protein ($P = 0.033$), fibrinogen ($P = 0.045$), ALP ($P = 0.000$), glucose ($P = 0.05$), and calcium ($P = 0.002$). Fibrinogen is an acute-phase reactant protein, stimulated by pro-inflammatory cytokines, and is a useful marker of inflammation in a variety of species.[8] Reference ranges in marsupials are typically from 1–4 g/L.[5] However, the time required for the protein concentration to become significantly increased, and the duration of the increased concentration, is based upon both the severity of inflammation and the species affected. Subadults had a higher fibrinogen concentration. Significant changes in fibrinogen concentration, in conjunction with an increase in neutrophils and altered neutrophil morphology, are often indicative of inflammatory change in macropodidae.[6] However, there were no signs of clinical disease or inflammation other than physiologic changes associated with immaturity, including an increased white cell, lymphocyte, and basophil count. When associated with an increased PCV and total protein concentration, the possibility of mild dehydration in the subadult cohort, leading to a relative increase in blood protein fractions, including fibrinogen, should be considered. The increase in serum calcium, relative to the adult cohort, may also reflect an increased total protein concentration resulting in a higher concentration of protein-bound calcium in circulation.

Increased serum ALP activity, associated with increased osteoblastic activity, occurs in all species.[25] The elevation in ALP in subadults is probably explained by the ALP isozyme in bone, which may cause an elevation of serum ALP in young growing animals.[23,24]

In relation to serum biochemistry, wild Gilberts potoroos had a greater total bilirubin ($P = 0.004$), urea ($P = 0.000$), creatinine ($P = 0.000$), globulin ($P = 0.002$), and phosphorus ($P = 0.000$) when compared to captive individuals. The relative increased urea, creatinine, globulin, and phosphorus concentrations may indicate a reduction in the glomerular filtration rate resulting from a prerenal, renal or postrenal cause. The most likely prerenal cause would be dehydration, as assessed through reduced skin turgor, dry mucosal surfaces, increased plasma protein concentration, a high hematocrit, and a high urinary specific gravity (USG). Unlike their captive counterparts, the wild population would not have continuous access to water and would maintain hydration through ingestion of tubers and truffles. In a study conducted concurrently by the authors, Gilbert's potoroo urinalysis values, including specific gravity, were analyzed using a one-way ANOVA; a mean of 1.021, with

a range of 1.004 and 1.039, was recorded with a standard error of 0.009 ($n = 35$). These values are similar to those found in companion animals.[28] A healthy, hydrated dog should have a USG between 1.015 and 1.045.[14] Extrapolating from these findings, the majority of Gilbert's potoroos were well hydrated. Furthermore, no statistical difference between captive and wild individuals was observed ($P = 0.19$) when assessing USG. Other renal or postrenal causes for the observed differences in the wild and captive population may include partial impairment of renal function due to renal disease, or to some other factor inhibiting the ability to retain water such as a partial deficiency or inhibition of ADH activity. Drug effects were not considered, as this population is not receiving any medical treatment. Inflammatory events leading to renal disease, such as *Treponema* infection, were considered. However, when blood values of *Treponema*-infected compared to non-*Treponema*-infected individuals were compared, no significant differences in urea, creatinine, phosphorus, and globulins were found. Furthermore, balanoposthitis caused by *Treponema* is present in both the captive and wild population.

The most likely explanation of the increased analytes in the wild population may be linked to the present lack of captive individuals with presumptive familial renal oxalosis. Renal oxalosis has been screened through urinary glycolate testing via DEC staff, and the captive population's diet has been modified to minimize oxalate consumption (D. Forshaw, pers. comm., May 2007). Renal oxalosis refers to the microscopic deposition of oxalate in the tubules, causing tubular epithelial necrosis and dysfunction.[18] Severe renal oxalosis was the cause of death of five animals in the captive population between 1994 and 2000. Four of these deaths occurred in one family group, with a high incidence in related individuals suggestive of an inherited condition (Horwitz and Forshaw, unpubl. data). Excess dietary oxalate intake was eliminated as a possible cause of the renal oxalosis. At present, a probable inherited disorder of oxalate metabolism, involving enzyme pathways digesting the intermediary substrate glyoxylate (degraded to oxolate and glycolate), is the most likely explanation (Horwitz and Forshaw 2001, unpubl. data). However, the possibility of other mechanisms cannot be dismissed. Renal oxalosis is presumed to still exist in the wild population, and these analyte values indicate that further urinary glycolate testing should be undertaken.

Elevated bilirubin was also evident in the wild compared to the captive cohort. Hyperbilirubinemia may be physiologic or pathologic and is caused by increased erythrocyte destruction, decreased uptake or conjugation of bilirubin by hepatocytes, disruption of bile flow, or increased enterohepatic circulation of bilirubin.[28] The wild cohort had statistically significant AST activity increases when compared to the captive population. However, increased CK activity was also present, muscle iso-enzyme induction was assumed, and hepatic hyperbilirubinemia was not suspected. The wild population did have a statistically significant lower PCV, Hb, and numbers of RBC compared to the captive population, which could be related to increased erythrocyte destruction. However, the lack of clinical signs of anemia, including pallor of mucous membranes, weakness, lethargy, and anemic cardiac murmurs, indicated a lack of clinical significance. Retention or fasting hyperbilirubinemia, due to anorexia or starvation, is the most-likely cause of the observed values, as wild individuals could potentially have been trapped 12 hr prior to anesthesia for blood collection and, given they are nocturnal, may not have eaten for the preceding 12 hr. Although once trapped the potoroos had access to the bait material, this was not always eaten. Captive potoroos were not fasted, and a fasting hyperbilirubinemia was not observed.

The captive population had a greater concentration of cholesterol compared to the wild population. However, no samples were grossly lipemic. Although the mean of the captive cohort was outside the upper 95% confidence interval of the mean cholesterol value in Gilbert's potoroos, this increase was thought to be dietary related rather than pathologic. All the captive Gilbert's potoroos were rated as well-conditioned to obese (body condition score 3.5 to 5). Wild potoroos had a body condition score of 2 and below. The diet of captive potoroos is based on the wild diet, where fungal material (truffles) were found to make up more than 90% of the diet.[20] However, owing to seasonal variation, the captive diet now consists of 10% (by weight) of hypogeal fungi[8] as well as mealworms, nuts, cereal, fruit, and vegetables. The fat content, especially from the mealworms and nuts, possibly accounts for the higher observed cholesterol concentration. Further investigation is warranted to prevent obesity-related health problems in the captive population.

The minimal hematology and biochemistry differences between the sexes is not surprising,

as numerous marsupial studies have reported no differences attributable to sex in the total leukocyte and differential leukocyte concentrations in common brushtail possums,[21] mountain brushtail possums,[29] or allied rock-wallabies.[26]

The minimal hematologic and biochemical differences between *Treponema*-infected and noninfected individuals is also not surprising, as the organism appears to be locally invasive, with no serologic response detected through using the TPPA, the EIA, and the RPR test. A venereal spirochetosis caused by *T. paraluis cuniculi* has been identified in rabbits. It results in vulval and preputial lesions, beginning as areas of hyperemia and edema, with the development of papules and vesicles followed by ulceration, crustiness, and hyperkeratosis.[9] This is very similar to the clinical presentation seen in Gilbert's potoroos. Like rabbits, potoroos did not seem to develop leukocytosis with *Treponema* infection, yet, unlike rabbits, they were nonreactive to the RPR test. This is the test currently used as a diagnostic aid for treatment success in the rabbit.[10]

The data from this study were used to create the first hematologic and biochemical reference intervals for Australia's most critically endangered mammal, the Gilbert's potoroo. Standardized capture, collection, and processing protocols were used to optimize data quality. These reference ranges will assist in the ongoing health management of the Gilbert's potoroo and will provide a useful comparison for other potoroid species, both in the wild and in captivity.

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