Health status of the red-billed tropicbird (*Phaethon aethereus*) determined by hematology, biochemistry, blood gases, and physical examination

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The red-billed tropicbird, *Phaethon aethereus*, is a species of seabird native to the Galápagos archipelago, and widely distributed across the neotropics. General health, blood chemistry, and haematology parameters have not been published for this species. Blood analyses were performed on samples drawn from 51 clinically healthy red-billed tropicbirds captured from their burrows at Islote Pitt on San Cristóbal Island in July, 2016 (21) and Daphne Major Island in June, 2017 (30). In the field, a point of care blood analyser (iSTAT) was used to obtain results for HCO_3^-, pH, pCO_2, pO_2, TCO_2, iCa, Na, K, Cl, Hb, HCT, anion gap, creatinine, glucose and urea nitrogen. Additionally, a portable Lactate Plus™ analyser was used to measure blood lactate, and blood smears were also created *in situ.* The blood slides were used to estimate leukocyte counts and 100-cell differentials. Alongside these biochemistry and haematology parameters, average heart rate, respiratory rate, body temperature and scaled mass index (calculated from weight and a body measurement) were compared to determine the standard measurements for a healthy individual. The baseline data, and reference intervals reported in this paper are essential to detecting changes in the health of red-billed tropicbirds in the future.

1   Health status of red-billed tropicbirds

2   **Health Status of the Red-Billed Tropicbird (*Phaethon aethereus*) Determined by Haematology,**

3   **Biochemistry, Blood Gases, and Physical Examination**

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20   Abstract: The red-billed tropicbird, *Phaethon aethereus*, is a species of seabird native to

21   the Galápagos archipelago, and widely distributed across the neotropics. General health, blood

22   chemistry, and haematology parameters have not been published for this species. Blood

23   analyses were performed on samples drawn from 51 clinically healthy red-billed tropicbirds

24   captured from their burrows at Islote Pitt on San Cristóbal Island in July, 2016 (21) and Daphne

25   Major Island in June, 2017 (30). In the field, a point of care blood analyser (iSTAT) was used to

26   obtain results for HCO_3^-, pH, pCO_2, pO_2, TCO_2, iCa, Na, K, Cl, Hb, HCT, anion gap, creatinine,

27   glucose and urea nitrogen. Additionally, a portable Lactate Plus™ analyser was used to measure

28   blood lactate, and blood smears were also created *in situ.* The blood slides were used to estimate

29   leukocyte counts and 100-cell differentials. Alongside these biochemistry and haematology

30   parameters, average heart rate, respiratory rate, body temperature and scaled mass index

31   (calculated from weight and a body measurement) were compared to determine the standard

32   measurements for a healthy individual. The baseline data, and reference intervals reported in

33   this paper are essential to detecting changes in the health of red-billed tropicbirds in the future.

34   **Key words:** Biochemistry, *Phaethon aethereus*, Galápagos Islands, red-billed tropicbirds,

35   haematology.

36   **INTRODUCTION**

37   The red-billed tropicbird (*Phaethon aethereus*) was traditionally considered a member of

38   the Pelecaniformes, in the family Phaethontidae (Livezey and Zusi, 2007; Bourdon *et al.*, 2008).

39   However, this placement has been revised with the rise of whole-genome analysis and tropicbirds

40   are now placed in their own order Phaethontiformes (Jarvis *et al.,* 2014). This long-lived pelagic

41   seabird is found across the neotropics, tropical Atlantic, eastern Pacific and the Indian Ocean

42 (Vilina *et al.,* 1994; Javed *et al*., 2008; Nunes *et al.,* 2017). The subspecies *P.a.mesonauta* (Orta,

43 1992; Nelson, 2005) breeds year-round in the Galápagos (Snow, 1965). This region holds one of

44 the largest colonies of this species, consisting of several thousand pairs on the Galápagos islands

45 and mainland Ecuador (del Hoyo *et al*., 1992; Orta *et al*., 2019). Other extant members of this

46 family include the white-tailed tropicbird and the red-tailed tropicbird.

47   To date, there has been a lack of information available on the health and population

48 trends for red-billed tropicbirds and related species. Among the Phaethontidae, only the red-

49 tailed tropicbird (*Phaethon rubricauda*) of Christmas Island, had leukocyte profiles reported

50 (Dehnhard *et al.*, 2011; Dehnhard and Henike*,* 2013). This study did not report on biochemistry

51 or blood gases, making our investigation the first of its kind for this family and the focal species.

52   The Galápagos Archipelagos are located 972km west of mainland Ecuador. They are a

53 sanctuary for breeding Pacific seabirds and are home to many endemic species. The islands are

54 impacted by climate change, introduced pathogens, parasites and invasive species (Wikelski *et*

55 *al*., 2004; Edgar *et al*., 2008; Riofrío-Lazo and Páez-Rosas, 2015; Dueñas *et al*., 2021). Due to the

56 many human-driven changes to the Galápagos, it is important to define 'clinically normal'

57 animals, so in the future, we can identify changes that occur in a species' health status (Valle *et*

58 *al.,* 2018). In this study, we provide a detailed account of 51 individuals anatomical, physiological,

59 and blood-specific values that are widely used to evaluate the health of wild seabirds (Work,

60 1996; Padilla *et al*., 2003; Padilla *et al*., 2006; Ratliff *et al*., 2017; Lewbart *et al*., 2017; Valle *et al*.,

61 2018; Valle *et al.,* 2020a; Valle *et al.,* 2020b). This work is also relevant because the global

62 population of red-billed tropicbirds are thought to be decreasing although the species is classified

63 as least concern (BirdLife International, 2019).

64   Ground-nesting birds, especially those on islands, are at risk from terrestrial predators,

65   particularly introduced non-native species (Stratton *et al.,* 2021). In the Galápagos, for example,

66   introduced predators such as pigs, rats, goats and cats threaten breeding birds (Cruz, 1987; Key

67   *et al.*, 1994; Riofrío-Lazo and Páez-Rosas, 2015; Debrot *et al*., 2014). Endemic island species are

68   also susceptible to diseases (Wikelski *et al*., 2004; Deem *et al*., 2011); in the Galápagos some have

69   been infected by introduced pathogens (Levin *et al*., 2012; Bastien *et al*., 2014; Tompkins *et al*.,

70   2017). Having baseline values from healthy animals provides a foundation for comparison when

71   diseased individuals are identified.

72   There has been no published work on the health of red-billed tropicbirds, but publications

73   have reported leucocyte profiles and heterophil to lymphocyte ratios for *P. rubricauda* (Work,

74   1996; Dehnhard *et al.* 2011; Dehnhard and Hennicke, 2013). Literature concerning red-tailed

75   tropicbirds on Christmas Island demonstrated leucocyte profiles are not influenced by sex or

76   breeding stage (Dehnhard and Hennicke, 2013). They also suggest that chicks of this species

77   invest heavily in innate immunity as they displayed significantly higher heterophil/lymphocyte

78   ratios than adults (Dehnhard *et al.*, 2011).

79   The aim of this study is to present baseline health results for the Galápagos Island red-

80   billed tropicbirds and to be able to identify changes in their health in future.

81   **Materials and methods**

82   This study was performed as part of a population health assessment authorized by the

83   Galápagos National Park Service (permit No. PC-57-16 and No. PC-59-17 to C.A. Valle) for ethics,

84   animal handling and permissions to work within the Galapagos Reserve (Valle *et al.*, 2018; Valle

85   *et al*., 2020a; Valle *et al*., 2020b; Tucker-Retter *et al*., 2021). The study was additionally approved

86   by the Universidad San Francisco de Quito ethics and animal handling protocol. Handling and

87   sampling procedures followed standard vertebrate protocols and veterinary practices. Data were

88   collected in 2016 at Punta Pitt, San Cristóbal Island (0°41' 59'' S; 89° 15' 09'' W) and in 2017 on

89   Daphne Major Island (0°25' 22.14 "S; 90°22' 16.17" W) from two separate multi-species seabird

90   colonies.

91   In June 2016 and June 2017, 21 and 30 adult red-billed tropicbirds, respectively, were

92   captured from their nesting holes, one at a time. At time of capture, the nesting status was

93   recorded, where possible, alongside the "age of chick" and "presence of eggs" or "other adult".

94   The adults were identified as distinct from the chicks by their plumage and bill colour. The birds

95   were restrained carefully by one handler, holding the bill closed, whilst supporting the weight of

96   the body and restraining the wings. The behaviour of the bird was monitored closely during

97   handling to ensure its safety. Within 10 minutes of capture, heart rate and respiratory rate were

98   recorded, as well as a blood sample quickly and safely drawn. This short turnaround time was to

99   reduce the potential effects of handling on blood chemistry values. The birds were then weighed,

100   using a Pesola® (Pesola, Prazisionswaagen AG, Schindellegi, Switzerland) measured for standard

101   body measurements, and had their cloacal temperature recorded using a digital thermometer.

102   All birds were deemed clinically healthy based on their behaviour, response to handling, and

103   physical examination by a veterinarian. Sex could not be distinguished since red-billed tropicbirds

104   are monomorphic (Nunes *et al.,* 2013).

105   Brachial vein puncture was performed using a heparinized 25-gauge needle and 1.0 ml

106   syringe to collect up to 1.0 ml of blood. To obtain the blood gas, electrolyte, and biochemistry

107   results, a drop of blood was used for instant analysis in the field, with a Portable iSTAT Clinical

108   Analyser (Heska Corporation, Loveland, Colorado, USA 80538). Different iSTAT cartridges were

109   used for different years, CG8+ vs CHEM8+, which meant the recorded health parameters differed

110   slightly. In 2017, pH, HCO_3^-, pCO_2 and pO_2 were not obtained. Another drop of blood was then

111   used to obtain lactate levels through a portable lactate analyser. To obtain total solids, a field

112   centrifuge was used to spin haematocrit capillary tubes for 3 minutes, and the plasma in the

113   capillary tubes was dropped onto a handheld refractometer. Calculated iSTAT haematocrits were

114   obtained for all individuals. In addition, blood smears were also created in the field, and were

115   later fixed and stained with Diff Quick (Jorgenson Laboratories, Loveland, CO, 80538 USA)

116   approximately 2 weeks after sampling.

117   The stained blood smears were used for the 100-cell white blood cell (WBC/leukocyte)

118   differentials and for WBC estimates (Work, 1996). The WBC differentials were obtained at a 100x

119   objective lens and completed by differentiating 100 white blood cells. This was recorded as a

120   percentage and these were used to calculate absolute values using the total leukocyte count

121   (estimated WBC count). Leukocytes were counted in 10 fields at a 40x objective. These were

122   averaged and multiplied by 2000 to obtain a leukocyte count per μL (Newman et al., 1997). Blood

123   smears from 2016 (N=21) and 2017 (N=30) were analysed by different observers, however, to

124   minimise observer differences, the subsequent observer was trained and supervised by the

125   observer from 2016.

126   Scaled mass index (Peig and Green, 2009; Peig and Green, 2010) was calculated using the

127   R package (standardized) major axis estimation and testing routines 'smatr' 3.4–8 (Warton *et al.*,

128   2011) was used for computing the scaling exponent using standardized major axis (SMA)

129   regression. Summary statistics were completed on the measured parameters. Normality was

130   tested using Shapiro-Wilk test and visualised through quantile-quantile plots. Despite 28 out of

131   the 34 variables of the variables being non-normal, the sample size is sufficiently large to invoke

132   the central limit theorem meaning linear regressions could be used to examine possible

133   relationships between SMI (scaled mass index) and log transformed blood biochemistry and

134   haematology differentials (Sainani, 2012). Sampling time was tested against the variables using

135   Kendall's rank correlation, a test chosen to handle non-normal data with outliers, reliably.

136   Significant results were then examined with linear regressions on log transformed data. A

137   standard α level of $P < 0.05$ was used for all statistical tests with R statistical software, version

138   3.6.2 (R Development Core Team) and figures were made using *ggplot2* (Wickham, 2009).

139   Reference intervals were calculated and are reported for health parameters.   They were

140   calculated using Reference Value Advisor Freeware (Geffré *et al*., 2011) with a sample number

141   <40 and >40, using robust methods, Box-Cox transforming data and removing outliers. Outliers

142   were identified through Tukey's testing and histograms, and emphasis was put upon retaining

143   rather than deleting data (Horn and Pesce, 2003). Alongside this,   the median, minimum and

144   maximum values have been made available as supplementary information to support in clinical

145   decisions (Friedrichs *et al.,* 2011; Klassen, 1999).

146   **Results**

147   In 2016 and 2017 a total of 51 adults of *P. aethereus* from two different colonies were

148   assessed; the blood chemistry, morphology and haematology results alongside reference

149   intervals are presented in Tables 1- 3.

150   The dominant leukocytes for P. *aethereus* at Islote Pitt and Daphne Major were

151   lymphocytes (μ =34.2%, absolute μ =995 x10^3/μL), eosinophils (μ =30%; absolute μ =938 x10^3/μL)

152   and heterophils (μ =34.1%, absolute μ =908 x10^3/μL) (Table 3). No basophils were observed in *P.*

153   *aethereus*, and monocytes percentages were low.

154   From the statistical analysis, sampling time was shown to demonstrate significantly strong

155   positive effects on pO^2 (P<0.05, tau=0.389) and strong negative impacts on pCO^2 (P<0.05, tau=-

156   0.367) and HCO^{3-} (P<0.05, tau=-0.361) as seen in Figure 1. A simple linear regression was

157   calculated to predict whether the calculated scaled mass index (SMI) affected the variables and

158   was graphically examined. Outliers were removed and SMI demonstrated no effects on the

159   variables.

160   Heterophils had a colourless cytoplasm with a high density of red to brown large fusiform

161   granules and a segmented lobed nucleus. Eosinophils had similar size to heterophils, with

162   amorphous to large granules and numerous small clear vacuoles, in accordance with *P.*

163   *rubricauda* (Work, 1996) for birds sampled from both populations.

164   No hemoparasites were observed for red-billed tropicbirds at either site.

165   **Discussion**

166   This is the first haematological profile of *P. aethereus*. This study reports an in-depth array

167   of morphometric, vital, blood biochemistry, and haematological parameters from red-billed

168   tropicbirds. No other studies present health assessments for this species of tropicbird and the

169   results from this study will be a reliable baseline for future health assessments. Reference

170   intervals for the health parameters are presented in this paper to ensure that there is information

171   to compare to if any further studies follow the population health of these tropicbird populations.

172   Healthy adult individuals were directly selected for this baseline health assessment and no sex

173   differential studies could be made with sufficient confidence since these birds are monomorphic

174   (Nunes *et al.,* 2013). Additionally, reproductive state was not always evident. Preanalytical

175   procedures for reference intervals usually including standardising the provisioning of the

176   individuals to be either fasted, or non-fasted, however this is not possible in wild populations. All

177   sample capture, handling, sample collection and processing were standardised. Outliers

178   identified by the Tukey's test were examined and only accepted after examining all the data from

179   the individual at once, as well as investigating a histogram of the transformed data. Identified

180   outliers were removed after this thorough investigating and before calculating the reference

181   intervals. Smaller sizes make this more difficult but the robust method is utilised as it puts less

182   emphasis on the extreme values (Horn and Pesce, 2003).

183   Leukocyte estimates for *P. aethereus* (μ= 2890 x10^3/μL, σ= 1380 x10^3/μL) were low

184   relative to other seabird species studied in the Galápagos during 2016 and 2017 at Daphne Major

185   and Punta Pitt, including great frigatebirds (μ=3700.37 x10^3/μL, σ=693.37 x10^3/μL), Galápagos

186   shearwaters (μ=3283 x10^3/μL, σ=6720 x10^3/μL), swallow-tailed gulls (μ=3928 x10^3/μL, σ=1098

187   x10^3/μL), and Nazca boobies (μ=17553 x10^3/μL, σ= 3333 x10^3/μL (Valle *et al*., 2018; Valle *et al*.,

188   2020a; Valle *et al*., 2020b; Tucker-Retter *et al*., 2021).

189   221.   The leukocyte differentials demonstrated no basophils and low to no monocyte

190   counts. These white blood cells are both uncommon in avian peripheral blood (Fudge,

191   1994). The leukocyte profiles showed almost equal percentage counts of heterophils,

192   lymphocytes and eosinophils. This percent of eosinophils is high relative to the eosinophil

193   percentage of other populations of *P. rubricauda* (Dehnhard *et al*, 2011; Dehnhard and

194   Hennicke, 2013; Luna *et al*., 2020). Eosinophils can vary greatly between avian species

195   therefore the differences noted here between the *P. aethereus* and *P. rubricauda* is not

196   unusual (Fudge, 1994) and the reported percentages presented in this paper are the

197   known baseline for this population.   When comparing other studies' leukocyte

198   differentials, as a percentage of 100 counted cells, Phaethontidae species have variable

199   percentage eosinophil counts. The population of red-billed tropicbirds in Galápagos had

200   a relatively high eosinophil percentage count (μ=30%), as well as the adult red-tailed

201   tropicbirds on Rapa Nui, which had μ=36.69% (Luna *et al.*, 2020). In contrast, the red-

202   tailed tropicbirds on Christmas Island, had very low eosinophil counts (μ=0-1%)

203   (Dehnhard *et al.*, 2011; Dehnhard *et al.*, 2013). Total leukocyte estimates and manual

204   differential counts have high coefficients of variation relative to automated nucleated cell

205   counts, which is a limitation of this study but was the only practical approach under

206   field conditions.

207

208   Stress is known to have adverse effects on wild animals (Sagar *et al.*, 2019; McMahon *et*

209   *al.*, 2005). Although this has never been studied in Phaethontiformes, handling time was kept

210   short to ensure minimal stress to the individual (Sorenson *et al.*, 2016).

211   When sampling time increased, pCO_2 and HCO_3 decreased, while pO_2 increased.

212   This is likely due to the birds' increased respiratory rate due to stress during capture and handling.

213   The changes in the blood chemistry can be explained through Le Châtelier's principle (Hopkins *et*

214   *al.*, 2022), with deep or rapid breathing drawing away more CO^2 and bringing in pO^2 more rapidly,

215   increasing the pO^2 levels and lowering the pCO^2. This disturbs the dynamic equilibrium of the

216   primary pH buffer system and the position of the equilibrium will shift to reestablish an

217   equilibrium, which is seen by the decrease in HCO^{3-}. If a change in pH was seen this could lead to

218   acute metabolic alkalosis (Fudge, 1994).

219   Seabird species in the Galápagos Archipelago are at risk since they are vulnerable to

220   human population growth on the Islands, tourism, and movement between the archipelago and

221   Ecuador (Walsh and Mena, 2016). Other pressures include introduced rodents predating ground-

222   nesting species in the Galápagos and although *P.aethereus* were not referenced in this study

223   (Harper and Carrion, 2021), other populations of this species face serious population decline due

224   to chick and egg predation from the rat species found in Galápagos, including both invasive house

225   (*Rattus rattus*) and brown rats (*Rattus norvegicus*) (Sarmento *et al.*, 2014).

226   Few health and physiology studies have been completed on red-billed tropicbirds due to

227   the animals' remote breeding habitat on steep rocky cliffs on remote islands. This study creates

228   a foundation of veterinary knowledge for this species and contributes to general knowledge of

229   seabird health. This information will be an essential comparison as increasing human presence

230   and pressure on this vulnerable ecosystem places small seabird rookeries at risk. Continuing to

231   monitor and understand species that live on the edge will be vital to their conservation and the

232   conservation of other coastal species.

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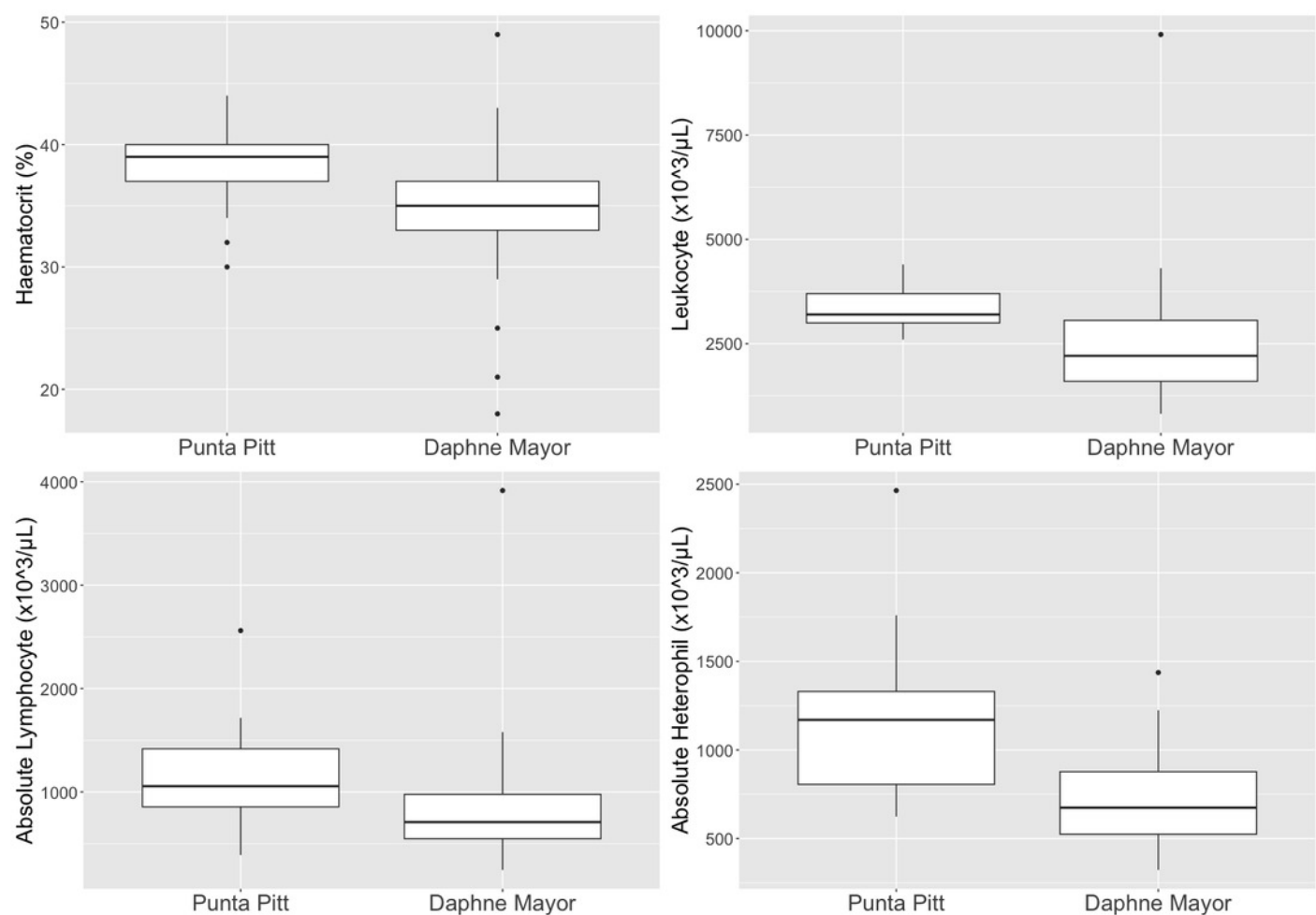
Figure 1

Blood Gases and Sampling Time Correlations.

Linear regression plots demonstrating significant correlations between sampling time and blood gases for red-billed tropicbirds (*Phaethon aethereus*) sampled in Punta Pitt (2016) and Daphne Major (2017).

Physiological measurements and blood sample time at Punta Pitt.

Summary of morphological and physiological measurements alongside sample time, which is time taken to obtain the blood sample from capture, reported as mean, standard deviation and range for 21 red-billed tropicbirds (*Phaethon aethereus*) at Punta Pitt, San Cristobal.

Table 1 summarising the mean, standard deviation, range and reference intervals for morphological and physiological measurements, alongside sample time, for 21 red billed tropicbirds (*Phaethon aethereus*) at Punta Pitt, San Cristobal, and 30 on Daphne Major.

Parameter	Red-billed tropicbird Values					Reference Values	
	μ	σ	median	range	n	reference intervals	missing (%)
Wing length (cm)	31.2	2.21	31.0	28.7-39.0	51	28.7 – 33.1	6
Tarsus length (cm)	3.20	0.501	3.14	2.20-4.96	51	2.25 – 3.96	2
Scaled Mass Index	504	186	444	265-1590	51	282 - 681	2
Weight (kg)	0.59	0.07	0.60	0.30-0.73	51	0.52 – 0.70	8
Respiratory rate (beats/min^{-1})	33.7	9.93	32.0	16.0-60.0	50	16.0 – 57.8	4
Heart rate (beats/min)	237	60.9	244	144-500	51	145.8 - 458	2
Body temperature (ºC)	39.6	1.0	39.6	37.7-41.6	51	37.8 – 41.6	0
Sample time (min:second)	7:98	6:32	6:00	2:00-38:00	46	N/A	N/A
Handling time (min:second)	15:00	6:55	13:00	5:00-40:00	48	N/A	N/A

Mean blood values at Punta Pitt.

Mean, standard deviation, and range for blood gas, biochemical and haematology values for 21 red billed tropicbirds (*Phaethon aethereus*) at Punta Pitt, San Cristóbal; M = manual and I = iSTAT

Table 2 Summarising the mean, standard deviation, range and reference intervals (including the interval and the percentage of values that were removed as outliers) for blood gas, biochemical and haematology values for 21 red billed tropicbirds (*Phaethon aethereus*) at Punta Pitt, San Cristóbal and 30 on Daphne Major.

Parameter	Red-billed tropicbird Values					Reference Values	
	μ	σ	median	range	n	reference intervals	missing %
HCTı %	36.4	5.62	37.0	18.0-49.0	50	25.9–47.9	4
Total Solids (g/L)	58	18.2	53.5	32-108	38	3.19-10.42	8
Haemoglobin (g/L)	124	191	126	61-167	50	99-150	8
pH (37◦C)	7.41	0.092	7.38	7.26-7.69	21	7.19-7.59	0
pH (Auto-corrected)	7.37	0.089	7.35	7.24-7.67	21	7.24-7.49	0
HCO3- (mmol/L)	17.4	3.41	16.8	11.9-25.4	21	11.7-26.23	0
pCO2 (mmHg)	27.7	6.12	27.8	13.3-37.3	21	12.3-39.0	0
pO2 (mmHg)	58.1	11.9	58.0	40.0-87.0	21	35.9-86.7	0
Na (mmol/L)	140	5.21	142	116-148	50	134–148	6
K (mmol/L)	3.48	0.699	3.40	2.40-6.60	50	2.44–4.08	6
iCa (mmol/L)	0.951	0.258	1.00	0.00-1.30	50	0.62–1.30	8
Glucose (mmol/L)	16.5	3.19	16.9	5.94-23.8	50	9.5–23.3	2
Lactate (mmol/L)	3.78	1.40	3.60	1.20-6.60	51	1.26–6.57	0

White blood cell count values for 21 red billed tropicbirds (*Phaethon aethereus*) at Punta Pitt, San Cristobal, and 30 of the same on Daphne Major.

Summarizing the mean, standard deviation, range and reference ranges for white blood cell count values for 21 red billed tropicbirds (*Phaethon aethereus*) at Punta Pitt, San Cristobal, and 30 on Daphne Major.

Table 3 summarising the mean, standard deviation, range and reference range for morphological and physiological measurements, alongside sample time, for 21 red billed tropicbirds (*Phaethon aethereus*) at Punta Pitt, San Cristobal, and 30 on Daphne Major.

Parameter	Red-billed tropicbird Values					Reference Values	
	μ	σ	median	range	n	reference intervals	missing %
Heterophil %	34.1	12.4	33.0	8.5-64.5	50	9.0-56.4	2
Monocytes %	1.66	1.32	1.50	0-4.5	50	0.0-4.5	0
Eosinophils %	30.0	16.8	28.0	2-68.0	50	3.1-66.5	0
Lymphocytes %	34.2	16.8	28.0	2.0-68.0	50	21.7-50.3	8
Basophils %	0	0	0	0-0	50	0-0	0
Estimated WBC x10^3/μL	2890	1380	3000	820-9910	50	830-4380	2
Absolute Values							
Heterophil (ABS) x10^3/μL	908	411	825	323-2460	50	323-2460	2
Monocytes (ABS) x10^3/μL	46.8	41.7	44.3	0-198	50	0-129	2
Eosinophils (ABS) x10^3/μL	938	841	684	64.8-4510	50	67.5-2160	8
Lymphocytes (ABS) x10^3/μL	995	610	862	246-3910	50	246-3910	4
Basophils (ABS) x10^3/μL	0	0	0	0-0	50	0-0	0