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Haematological reference intervals in captive African Grey parrots (*Psittacus erithacus*)

*Helena Gaspar[1], Ferran Bargallo[2], Jordi Grifols[2], Elisete Correia[3], Maria de Lurdes Pinto[4]**

[1]*Animal and Veterinary Research Centre (CECAV), Trás-os-Montes e Alto Douro University, Quinta de Prados, Vila Real, Portugal*
[2]*Zoològic Veterinaris, Carrer de la Conquista, Badalona, Barcelona, Spain*
[3]*Mathematics Departament, Trás-os-Montes e Alto Douro University, Vila Real, Portugal*
[4]*CEMAT/IST-ID, Center for Computational and Stochastic Mathematics, Instituto Superior Técnico, University of Lisbon, Lisbon, Portugal*

**Corresponding author: lpinto@utad.pt*

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Abstract: The African Grey parrot (*Psittacus erithacus*) is one of the most popular avian pets in exotic animal practice. In this study, blood samples (n = 459) from healthy African Grey parrots (n = 239) were collected for haematological testing. Reference intervals were determined according to the guidelines of the American Society of Veterinary Clinical Pathology with the Reference Value Advisor (v2.1) freeware. The calculated reference intervals (RIs) were: packed cell volume 38–59%; haemoglobin 105–154 g/l; RBC 2.3–3.3 10^6/µl; MCV 145.5–199.7 fl; MHC 38.3–55.6 pg; MCHC 232–313 g/l; WBC 2.7–12.6 10^3/µl; heterophils 1.2–10.2 10^3/µl; lymphocytes 0.8–4.3 10^3/µl; monocytes 0.0–0.6 10^3/µl; basophils 0.0–0.1 10^3/µl; eosinophils 0.0–0.2 10^3/µl. To the best of our knowledge, this is the first study in psittacines to use such a high number of birds and samples to establish haematological species-specific RIs, providing a valuable tool to professionals dealing with the health of African Grey parrots.

Keywords: avian medicine; blood cell count; haematology

Establishing clinical pathology databases for a particular species, including haematological reference intervals (RIs), is a fundamental tool to diagnose, monitor and screen diseases. However, it is widely known that establishing reference intervals in exotic and wild avian species is a challenge. In these species, RIs are particularly important, since they tend to mask clinical signs, and an early diagnose of an illness may by crucial to the health status and survival of an avicultural collection. Most sources suggest a broad range of values, probably due to the fact that they do not take variability factors into account that can impact the haematological parameters. The current reference intervals (RIs) for avian red blood cells (RBCs), packed cell volume (PCV) and haemoglobin (Hb) vary significantly among reports and among the species sampled (Fudge 2000a). The morphological traits, feeding ecology, living habitat, and migratory behaviour, to name a few, can certainly account for some of the differences observed in the haematological RIs reported for avian species. A significant part of this varia-

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tion can also be attributed to the different sample collection, preparation, and storage techniques, as well as to the use of automated or manual methods. After all, RIs are specific to a particular set of conditions (Friedrichs et al. 2012).

The haematological RIs commonly used in clinical practice for African Grey parrots do not state important details regarding the individuals sampled and methods used (Fudge 2000b; Marion and Carpenter 2013), which are common sources of variability when defining haematological RIs. One of the sources does not list the used methodology to acquire such data, nor the number of animals, the sex, age, health status or if sedation was used. In the second source, the only known aspect regarding the data is the "number of normal animals", which is 495, and it does not provide any additional information about the methodology, sex, age, health status or if sedation was used (Fudge 2000b). Furthermore, it is not clarified what is considered a "normal animal".

It is important to refer to the fact that RIs may not accurately reflect the distribution of the haematological values in a healthy population, because most are established using indirect sampling which inadvertently includes unhealthy individuals, and, therefore, is more susceptible to error than direct sampling, in which the criteria are established prior to selecting the individuals (Friedrichs et al. 2012). In order to obtain reliable results, all the individuals included in this study were considered healthy at the time of the blood collection, through inclusion criteria such as a physical examination, biochemistry parameters and a coprology analysis. The data, such as age and sex of the sampled individuals, were also collected to further characterise the population of African Grey parrots under study.

MATERIAL AND METHODS

Animals

Blood samples (n = 459) were collected from African Grey parrots (*Psittacus erithacus*) examined in the first consultations or check-ups between March, 2009 and July, 2017 at the Zoològic Veterinaris (Barcelona). The selection of animals included in this study followed the guidelines suggested by the American Society of Veterinary Clinical Pathology (ASCP) (Friedrichs et al. 2012). Animals from both sexes (n = 239) were selected based on their health status, confirmed by the inclusion criteria, such as the physical examination (attitude, body weight, thoracic auscultation, hydration status), biochemistry indicators (urea, uric acid, total protein, γ-glutamyl transferase, creatine kinase, aspartate aminotransferase, calcium, and phosphorus) and coprology analysis. The exclusion criteria included any sign of disease or the recent administration of pharmacologically active agents, with the exception of midazolam used for sedation purposes during sampling. The data regarding the age, sex, and diet of the animals were obtained either by the clinical history or by the physical examination. The age of the animals is expressed as a mean and standard deviation (M ± SD). The mean age of the sampled animals (n = 239) was 5.57 (± 5.15) years old. There were 101 males (42.26%) and 92 females (38.49%). The average age of the males and females was 5.46 (± 4.58) and 5.20 (± 4.88) years old, respectively. It was not possible to retrieve the information regarding the sex in 46 (19.25%) of the sampled animals. Concerning the diet, 116 animals (48.54%) were fed a good quality commercial feed supplemented with daily fruits and vegetables, 2 (0.84%) were fed with a good quality commercial feed, but without supplementation. Sixteen animals (6.69%) were only fed mixed seeds as their primary diet and 7 (2.93%) were fed with the same primary diet with additional daily fruits and vegetables. Ninety-eight animals (41.00%) had no information in the clinical file concerning their dietary habits.

Blood sample collection, blood film and haematological testing

The animals were carried out in their carrier or cage to the hospitalisation area and gently, but quickly, restrained with a disposable underpad by the neck, forming an Elizabethan grip. In the cases in which sedation took place, 1 mg/kg of midazolam (Midazolam Normon EFG®, 15 mg/3 ml; Normon S.A., Madrid, Spain) was administered intranasally. The animals were then returned to the cage, covered with an underpad. After waiting for 10 min for the midazolam to take effect, the birds were moved to the examination table, and laid down horizontally, while maintaining the same Elizabethan grip. The phlebotomy was performed mostly in the right jugular vein as previously described (Best 2005; Chitty

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2005; Clark et al. 2009; Tully 2009; Bellwood and Andrasik-Catton 2014; Campbell 2015a; Campbell 2015b; Doneley 2016a). Blood was collected into heparin and EDTA tubes. Blood films were made using the whole blood without an anticoagulant and were air dried before staining. The haematological testing was usually undertaken the same day of the blood collection and was performed to determine the packed cell volume (PCV), haemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), total erythrocyte count (TRBC), total leukocyte count (TWBC), and differential leukogram with absolute and relative counts. All the haematological testing was performed in an in-house laboratory as described in the following paragraphs. To keep the variation to a minimum and in order to provide usable data, as recommended by the (ASCP) guidelines regarding preanalytical and analytical procedures (Friedrichs et al. 2012), all the materials and methodology remained constant throughout the study period, and the operator carrying out the laboratory procedures was always the same.

The blood film staining was performed with a quick stain (Jorvet Dip Quick Stain; Jorgensen Labs, Inc., Loveland, CO, USA). Each solution was stored in a sterile container. The staining procedure was adapted from Campbell (2015a), and Bellwood and Andrasik-Catton (2014). The air-dried slide was dipped into the methanol fixative solution five times (1 sec each time), and the excess product was allowed to drain; the slide was dipped into the first staining solution five times (1 sec each time), and the excess product was allowed to drain; the slide was finally dipped into the second solution five times (1 sec each time), and the excess product was allowed to drain, the slide was then rinsed with distilled water and allowed to air dry. Depending on the blood film thickness or the quality of the staining at the end of this process, the slide would be further stained if necessary.

Packed cell volume (PCV)

To determine the PCV, a capillary tube with well-mixed anticoagulated blood (from the EDTA tube) was filled to ¾ and one end of the tube was sealed with clay. The tube was centrifuged in a microhaematocrit centrifuge (DGMH-24; Diagnostic Grífols, S.A., Barcelona, Spain) for 5 min at 9 520 *g*. The tube was then properly aligned in the haematocrit reader (Gri-Cel, S.A., Barcelona, Spain) and the value of the PCV was determined.

Total cell count: Erythrocytes and leukocytes

The total red and white blood cell counts were determined using a manual method adapted from Campbell (1994), Samour (2006) and Campbell (2015c). The dilution ratio used was 1 : 200. To dilute the blood, whole blood from the EDTA tube was drawn to the 0.5 mark of a diluting pipette and then to the 101 mark with a Natt and Herrick's solution (Vetlab, Palmetto Bay, FL, USA). After waiting for 2 min for the blood and solution to mix, the improved Neubauer chamber haemocytometer (Brand®) was covered with a coverslip, and one side of the haemocytometer was filled with the blood and stain solution by capillary action. The cells were allowed to settle for 5 minutes. The blood cells were observed under the light microscope (BA210E Trinocular; Motic®, Carlsbad, CA, USA) and the RBC within the four corners and central squares (each one consisting of 16 smaller squares) as well as the ones that overlapped the top and left border of the Neubauer haemocytometer were counted (10 × objective, 100 × magnification); the obtained number was multiplied by 10 000 to reach the total number of RBC per microlitre (or cubic millimetres). The WBC within the nine larger squares on the grid, as well as all the cells that overlap the top and left border were counted using the same objective. 10% was then added to the obtained value, and then it was all multiplied by 200:

$$\text{WBC/mm}^3 = (\text{total leukocyte in 9 squares} + \text{+ 10\% of total WBC}) \times 200 \quad (1)$$

These counts were repeated on the second side of the haemocytometer, and the counts from both chambers were averaged.

Haemoglobin concentration (Hgb)

To determine the haemoglobin concentration, a quantitative method was used in this study. The haemoglobin was oxidised into cyanmethaemo-

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globin by a series of reactions and then measured by spectrophotometry (Campbell 2010). Drabkin's colorimetric solution was used as the reagent (Spinreact 2012). The spectrophotometer (M100; Boehringer Ingelheim, Ltd, Biberach, Germany) was adjusted to zero with a blank solution (distilled water) at 540 nm. Whole uncoagulated blood (20 µl) was pipetted into a cuvette with 40 µl of Drabkin's solution (Spinreact, Girona, Spain). To allow for the conversion, the solution was mixed and incubated at room temperature (15–25 °C) for 3 min after which the absorbance of the sample was read at 540 nm and the haemoglobin concentration was determined.

Red blood cell indices

After the primary haematologic parameters (packed cell volume, erythrocyte count and haemoglobin concentration) had been determined, the secondary haematological parameters were obtained using the following formulas (Clark et al. 2009):

$$\text{MCV (fl)} = \frac{\text{PCV (l/l)}}{\text{RBC count } (10^6/\mu\text{l})} \times 1\,000 \quad (2)$$

where:
MCV – mean corpuscular volume;
PCV – packed cell volume;
RBC – red blood cell.

$$\text{MCH (pg)} = \frac{\text{Hb (g/l)}}{\text{RBC count } (10^6/\mu\text{l})} \quad (3)$$

where:
MCH – mean corpuscular haemoglobin;
Hb – haemoglobin;
RBC – red blood cell.

$$\text{MCHC (g/k)} = \frac{\text{Hb (g/l)}}{\text{PCV (l/l)}} \quad (4)$$

where:
MCHC – mean corpuscular haemoglobin concentration;
Hb – haemoglobin;
PCV – packed cell volume.

White blood cell differential count

To determine the white blood differential count (Campbell 1994; Samour 2006; Bellwood and Andrasik-Catton 2014; Campbell 2015c), the monolayer area in the blood film was examined under a microscope (BA210E Trinocular; Motic®, Carlsbad, CA, USA) with a 100 × immersion oil objective. The slide was scanned in a methodical grid pattern, in order not to count the same area twice. As a routine, 100 WBC were counted (if the total WBC was increased, 200 WBC would be counted to maintain accuracy). The relative WBC count was obtained by using the following formula (if 200 cells were counted, the division would be by 200):

$$\text{Cell type (\%)} = \frac{\text{Number of cell type observed}}{100} \quad (5)$$

The absolute white blood cell count was determined using the following formula (if 200 cells were counted, the division would be by 200):

$$\text{Cell type} \times 10^3/\mu\text{l} = \frac{\text{Relative (\%)}}{100} \times \frac{\text{WBC count}}{(10^3/\mu\text{l})} \quad (6)$$

where:
WBC – white blood cell.

Statistical analysis

Reference intervals were determined using an Excel (Excel; Microsoft Corp., Redmond, WA, USA) spreadsheet with the Reference Value Advisor (v2.1) freeware that performs computations following the IFCC-CLSI recommendations suggested by the ASVCP guidelines (Geffre et al. 2011; ASVCP 2012). The set of calculations included common descriptive statistics (mean, median, SD, minimum, and maximum values), as well as the Anderson-Darling test and a visual evaluation of the data histograms to assess the normal distribution of the variables. Dixon and Tukey's tests were used to identify the outliers. Those classified as "suspect" were kept. The reference intervals were determined using non-parametric methods since the data distribution was not Gaussian [P (Anderson-Darling) < 0.05].

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Table 1. Haematological RIs for the captive African Grey parrots (*Psittacus erithacus*)

Parameter	SI Units	*n*	Means	Median	SD	Min	Max	RI	Lower ref lim 90% CI	Upper ref lim 90% CI	Distribution	Method
PCV	%	459	47.8	47.5	4.8	35.5	65.0	38–59	37–39	57–60.5	NG	NP
Haemoglobin	g/l	459	128	127	12	58	170	105–154	103–106	150–158	NG	NP
RBC	10^6/µl	459	2.8	2.8	0.3	2.0	4.0	2.3–3.3	2.2–2.3	3.3–3.4	NG	NP
MCV	fl	459	172.6	172.0	14.8	139.3	243.2	145.5–199.7	143.8–148	196.2–207.2	NG	NP
MCH	pg	459	46.2	45.7	4.8	22.7	70.9	38.3–55.6	37–38.9	54.3–57.8	NG	NP
MCHC	g/l	459	268	267	21	130	342	232–313	229–235	306–319	NG	NP
WBC	10^3/µl	459	6.1	5.6	2.3	2.1	16.2	2.7–12.6	2.6–3.1	11–13.9	NG	NP
Heterophils	10^3/µl	455	3.8	3.4	2.1	0.59	13.77	1.2–10.2	1–1.3	8.4–10.7	NG	NP
Lymphocytes	10^3/µl	459	2.1	1.9	0.9	0.23	5.54	0.8–4.3	0.7–0.8	4.0–4.6	NG	NP
Monocytes	10^3/µl	459	0.1	0.2	0.1	0.0	1.76	0.0–0.6	0.0–0.0	0.5–0.7	NG	NP
Basophils	10^3/µl	459	0.0	0.0	0.0	0.0	0.32	0.0–01	0.0–0.0	0.0–0.1	NG	NP
Eosinophils	10^3/µl	459	0.0	0.0	0.0	0.1	0.3	0.0–0.2	0.0–0.0	0.1–0.2	NG	NP

max = maximum; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; MCV = mean corpuscular volume; min = minimum; *n* = number of samples; NG = non-Gaussian; NP = non-parametric; PCV = packed cell volume; RBC = red blood cell; ref lim = reference limit; SD = standard deviation; WBC = white blood cell

RESULTS

The calculated RIs for the haematological variables of the clinically healthy African Grey parrots (*Psittacus erithacus*) are listed in Table 1. Four outliers were excluded from the heterophil count. No haemoparasites or morphological differences were observed in the RBCs and WBCs.

DISCUSSION

A blood analysis is generally considered a lesser invasive method to gather insight into the health status of an individual, when compared to other auxiliary diagnostic methods. It is especially important in avian medicine because these animals tend to hide signs of disease and, when present, they are often non-specific (Campbell 2015b; Doneley 2016b). Regarding the haematology of the African Grey parrot (*Psittacus erithacus*), which is one of the most frequently encountered psittacine species in avian medicine, the data from the literature is rather limited or incomplete. In the present study, the haematological values of healthy specimens of captive African Grey parrots were assessed in order to establish the haematological RIs for captive animals of this species. This is the first study in psittacines that uses such a high number of animals (*n* = 239) and samples (*n* = 459) to determine species-specific RIs. In order to obtain reliable results, all the individuals included in this work were considered healthy at the time of blood collection, through inclusion criteria such as a physical examination, biochemistry parameters and a coprology analysis. The average age of the individuals included in this study was 5.57 years old, being fairly similar for males (5.45 years old) and females (5.16 years old), which is relevant because the African Grey parrot reaches sexual maturity between the ages of 4 and 6 (Marion and Carpenter 2013). In this study, the great majority of haemograms belonged to sexed individuals and the number of haemograms per sex was similar, making the sample more consistent. Additionally, to further characterise the population of the sampled animals, data regarding their diet were also collected. The effect of the diet in the haematological parameters in avian species has not been comprehensively studied, but a study demonstrated that adding black seed (*Nigella sativa*) significantly increased the RBC count, Hgb concentration and PCV, probably due to its hepatoprotective qualities (Toghyani et al. 2010). However, a study conducted in budgerigars (*Melopsittacus undulatus*), where one group was fed a mixed seed diet and another was fed a balanced formulated diet, found no significant differences in the haematological values (Fischer et al. 2006). In the present study, the majority of the samples were from animals fed with

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a good quality primary diet (54.03%) and animals with mixed seeds as their primary diet (7.63%).

There are several aspects that can impact the results from either manual or automatic methods. When using manual methods, one of the most prominent sources of variation or errors is of human origin. These include miscounting, underestimating or overestimating the cell types in the differential counting, as well as variations in the sample preparation, collection and storage (Fudge 2000b; Marion and Carpenter 2013; Campbell 2015d). These can be reduced, however, by using the same equipment, methodology and human operator (Fudge 2000c; Campbell 2015c). In the present work, the methodology and equipment were constant throughout the study period, and all the manual methods used to determine the haematological values were performed by the same experienced operator. This is relevant because if the operator is overvaluing or undervaluing something, that error will be constant throughout the samples and parameters. Moreover, the manual methods used in this study are considered the gold standard in avian haematology, due to the differences encountered in avian cell morphology, namely, nucleated erythrocytes (Campbell 2015b; Campbell 2015d; Doneley 2016b). Additionally, other studies also provide evidence that a haematologic analysis can be performed in an in-clinic scenario with satisfactory results (Rishniw and Pion 2016).

Regarding the red blood cell parameters, it should be noticed that the values of the MCV presented 30.72% of the samples above the RIs, which suggests that a substantial part of the population may have larger erythrocytes than what is established, which is not necessarily pathological (Bearhop et al. 1999), as the other red blood cell values were within the established RIs for the species (Marion and Carpenter 2013). The average TWBC count was low compared to the reported RIs (Marion and Carpenter 2013), which indicates that many haemograms were lower within the range. This could be a consequence of the number of juvenile animals included in the sample. In fact, the lower results from the younger animals are probably responsible for the 33.33% of the samples presenting leukopenia according to the previously reported RIs, since almost half of the sampled haemograms belonged to juvenile animals. The RI values for the TWBC in African grey parrots were much higher in a study using indirect sampling, which was attributed to stress or inflammatory conditions that are not abrogated by an indirect method. However, the RIs reported by the same authors when the samples were obtained from clinical normal birds were similar to those obtained in the present study (Tang et al. 2013). These results further reinforce the importance of properly defined species-specific RIs, otherwise healthy newborn and young animals may be considered immunosuppressed when their values are normal for their age group. Regarding the distribution of the differential counts of the leucocytes, the results suggest that the African Grey parrot is a predominantly heterophilic species, displaying a higher circulation value of heterophils when compared to lymphocytes, which is in agreement with previous reports (Fudge 2000b; Marion and Carpenter 2013). However, within the heterophil values, there was a considerable amount of haemograms above (15.90%) and below (15.03%) the reported RIs for this species (Marion and Carpenter 2013) and further studies are required to elucidate this broad variation. All the animals included in this study lived in captivity and several animals were tested in different seasons of the year. The effect of the season was tested, but no significant differences were found. The influence of captivity on the haematology has not been the subject of many scientific papers. In a study conducted in blue-fronted amazons (*Amazona aestiva*), comparing the haematological values in free-ranging and captive birds, the values of the lymphocyte and monocyte counts were higher in the captive birds, whereas the WBC, heterophil and eosinophil counts were significantly higher in the free-ranging birds (Deem et al. 2005). The higher WBC and heterophil count was probably associated with the stress of the capture (Campbell 2015b). The results obtained in this study further highlight the need to establish both species-specific RIs and RIs for different demographics. The demographics of the reference population should be representative of the patient population and while the latter is known, the reference population is not. Unless the RIs are representative of the patient's demographic and are determined using similar pre-analytical procedures and comparable analytical methods, they are not appropriate as a diagnostic reference for clinical decision-making, particularly when using indirect sampling methods.

Although they may be useful in the absence of other data, the generation of RIs through this meth-

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od only produces rough estimates, and it was considered not suitable for the psittaciformes species (Tang et al. 2013). Interpreting clinical data using inappropriate RIs may lead to the misclassification of a patient, which can result in the misdiagnosis, improper treatments or both, which, in avian medicine, could compromise an entire avicultural collection.

Conflict of interest

The authors declare no conflict of interest.

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