Anemia is a common clinicopathologic abnormality detected in avian species. It has been estimated that the frequency of anemia in birds, based on samples submitted for CBCs, is 12.7%. The initial diagnostic approach to anemia in both mammalian and avian patients is to first classify the anemia as regenerative or nonregenerative on the basis of the quantity of reticulocytes. For several mammalian species, reticulocyte counts are quantitatively assessed by use of automated analyzers. In contrast,avian erythrocyte regenerative responses are evaluated via semiquantitative assessment of polychromasia or estimation of reticulocyte percentages. Reticulocyte staining was performed in all samples by use of a 1:3 (whole blood to new methylene blue) dilution with incubation for 10 minutes at room temperature (approx 22°C); reticulocytes were assessed as a percentage of 1,000 RBCs by 2 independent observers. In Wright-Giemsa–stained blood smears, a polychromatophil percentage was similarly determined.

Results—4 avian reticulocyte types were defined: ring-form reticulocytes, aggregate reticulocytes, and 2 subcategories of punctate reticulocytes. A reticulocyte-staining protocol was optimized. Interobserver and intraobserver variations in assessment of reticulocyte and polychromatophil percentages were not significant. A strong positive correlation (Spearman coefficient of rank correlation \( \rho = 0.978 \)) was identified between the percentage of polychromatophils and the percentage of ring-form reticulocytes.

In addition, to the authors’ knowledge, a quantitative correlation between polychromatophils and various reticulocyte morphologic types in avian species has not been published.

In studies performed throughout the 1900s and in recent years, a variety of methods for counting avian reticulocytes has been used, and often the reports fail to define the method of reticulocyte counting. Additionally, the terms reticulocyte and polychromatophil are sometimes used interchangeably. This lack of uniformity in avian reticulocyte assessment prevents comparison of results among studies and has been identified as a likely cause of apparent variation in reticulocyte counts in chickens.10 A similar lack of uniformity in avian reticulocyte assessment exists among veterinary diagnostic laboratories. Many laboratories report only a semiquantitative estimate of polychromasia, whereas others report reticulocyte percentages (derived by use of various methods); few laboratories have established reference intervals for either variable.

The purpose of the study reported here was to develop a reticulocyte classification scheme, optimize an avian reticulocyte staining protocol, and compare the percentages of reticulocyte types with polychromatophil percentage in blood samples from birds. Additionally, determination of a reference interval for polychromatrophils in psittacines was undertaken.

Materials and Methods

Definition of reticulocyte types—Four types of reticulocytes were defined on the basis of the reticulum pattern revealed via supravital NMB staining (Figure 1). The reticulocyte classification categories were as follows: punctate-1 (<10 dots of stained reticulum); punctate-2 (≥10 dots of stained reticulum); aggregate (any aggregates of stained reticulum, distributed diffusely within the cytoplasm); and ring-form (aggregates of stained reticulum that formed a ring—contiguous or discontinuous—around at least half of the reticulocyte nucleus).

Comparison of reticulocyte-staining methods and evaluation of sample storage—A single 4-mL sample of whole blood was collected (immediately prior to euthanasia) from an injured red-tailed hawk that was brought to the veterinary medical hospital at the University of California, Davis; EDTA was used as the anticoagulant for the sample. The whole blood sample was diluted with NMB stain (1:1, 1:2, 1:3, and 1:4 [blood to stain]). Whole blood volumes of 10 to 20 μL were placed into plastic bullet tubes, and appropriate volumes of stain were added. The tubes were capped, mixed by repeated inversion, and allowed to incubate at room temperature (approx 22°C) for 5, 10, 30, or 60 minutes. Repeated inversion of the tubes was again performed before a direct smear of the contents of each was made. Sixteen smears (1 from each of the 4 diluted samples that underwent each incubation period) were evaluated by 1 observer (MPS). By use of the aforementioned reticulocyte classification scheme, the percentage of each reticulocyte type among 1,000 RBCs examined on each smear was determined.

A dilution of 1:3 (blood to stain) and a 10-minute incubation time were used to evaluate reticulocyte staining after 4, 12, 24, and 48 hours of storage of the EDTA whole blood sample at 4°C. Staining quality was subjectively evaluated for all smears, and the percentage of each reticulocyte type among 1,000 RBCs examined on each smear was determined. Degenerated erythrocytes, defined as cells with fragmented nuclear chromatin or deeply basophilic cytoplasm (or both), were counted separately when observed.

Comparison of polychromatophil and reticulocyte percentages—Samples of blood (anticoagulated with EDTA) from 31 birds that were examined at the
Descriptive statistics were calculated (ie, IECs were excluded from polychromatophil and total RBC counts) to express polychromatophils as a percentage of the combined number of polychromatophils and mature RBCs. A combined percentage of polychromatophils and IECs, which expressed all immature erythrocytes as a percentage of all erythroid cells, was also calculated. The 4 reticulocyte types (punctate-1, punctate-2, aggregate, and ring-form) were each counted in the NMB-stained smears and expressed as percentages. A combined percentage of aggregate and ring-form reticulocytes was also calculated.

Comparisons were made between adjusted polychromatophil percentage and percentages of each of the 4 reticulocyte types and also the combined percentage of aggregate and ring-form reticulocytes. Comparisons were also made between the combined percentage of polychromatophils and IECs and percentages of the 4 reticulocyte types and also the combined percentage of aggregate and ring-form reticulocytes. To evaluate the potential for observer bias, the differential counts on 1,000 cells were repeated on all NMB-stained smears by both observers at the conclusion of the study.

Generation of a reference interval for polychromatophils in healthy psittacines—Clinic records from January 2003 to January 2007 were searched electronically for psittacines that underwent a routine wellness examination. A bird was considered healthy if no clinical abnormalities or other evidence of disease was detected via physical examination and if results of a CBC and plasma biochemical analyses were within published reference intervals for healthy psittacines.2 Birds were excluded if they did not meet all of those criteria or if they were < 6 months old. Differential counts on 1,000 RBCs were performed on archived Wright-Giemsa-stained smears of blood from the birds that met the criteria for inclusion. Percentages of polychromatophils and IECs were recorded for each smear. Absolute polychromatophil counts were calculated by multiplying the polychromatophil percentage by the total RBC count (manual leukocyte counts were subtracted from the total nucleated cell count obtained from the hematology analyzer2 to obtain the total RBC count).

Statistical analysis—Descriptive statistics were generated for data from the comparison of staining methods.4 Interobserver and intraobserver variation in the percentage of ring-form reticulocytes was assessed via repeated-measures ANOVA.5 Polychromatophil and reticulocyte percentages were compared by use of a Spearman rank correlation procedure.6 Deming regression was used to compare polychromatophil and ring-form reticulocyte percentages recorded by both observers.6 The distributions of both the percentage and absolute polychromatophil data were evaluated by use of the D’Agostino-Pearson test.4 A value of P < 0.05 was considered significant.

Results
Comparison of reticulocyte-staining methods and evaluation of storage—Staining quality of the 4 smears made from blood samples that were diluted 1:1 with NMB stain and incubated at room temperature for 5, 10, 30, or 60 minutes was unacceptable for differential counting because the reticulum was often faintly stained, thereby precluding differentiation of various reticulocyte types. Staining quality of the remaining 12 smears was assessed as acceptable for differential

<table>
<thead>
<tr>
<th>Variable</th>
<th>Punctate-1† (%)</th>
<th>Punctate-2† (%)</th>
<th>Aggregate† (%)</th>
<th>Ring-form† (%)</th>
<th>Aggregate and ring-form (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polychromatophil and IEC (%)</td>
<td>-0.490</td>
<td>-0.192</td>
<td>0.377</td>
<td>0.978</td>
<td>0.651</td>
</tr>
<tr>
<td>Polychromatophil and IEC (%)</td>
<td>(0.026)</td>
<td>(0.014)</td>
<td>(0.019)</td>
<td>(&lt; 0.001)</td>
<td>(&lt; 0.001)</td>
</tr>
</tbody>
</table>

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<th>Variable</th>
<th>Punctate-1† (%)</th>
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<th>Ring-form† (%)</th>
<th>Aggregate and ring-form (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polychromatophil and IEC (%)</td>
<td>-0.498</td>
<td>-0.180</td>
<td>0.370</td>
<td>0.976</td>
<td>0.647</td>
</tr>
<tr>
<td>Polychromatophil and IEC (%)</td>
<td>(&lt; 0.001)</td>
<td>(0.166)</td>
<td>(0.020)</td>
<td>(&lt; 0.001)</td>
<td>(&lt; 0.001)</td>
</tr>
</tbody>
</table>

Direct smear were prepared from samples that were stained with NMB stain in a 1:3 dilution (blood to stain) and incubated at room temperature (approx 22°C) for 10 minutes. Wright-Giemsa-stained smears were also available from routine CBCs performed for each bird. Two observers each performed differential counts on 1,000 cells on the Wright-Giemsa–stained smears and a direct smear was prepared. Two observers (JLJ and MPS [observers 1 and 2]) each performed differential counts on 1,000 cells on the Wright-Giemsa– and the NMB-stained smears. Normochromic erythrocytes, polychromatophilic erythrocytes, and IECs (defined as round erythroid cells with basophilic cytoplasm) were counted in the Wright-Giemsa–stained smears (Figure 2). For comparison of different combinations of cells to reticulocytes, an adjusted polychromatophil percentage was calculated (ie, IECs were excluded from polychromatophil and total RBC counts) to express polychromatophils as a percentage of the combined number of polychromatophils and mature RBCs. A combined percentage of polychromatophils and IECs, which expressed all immature erythrocytes as a percentage of all erythroid cells, was also calculated. The 4 reticulocyte types (punctate-1, punctate-2, aggregate, and ring-form) were each counted in the NMB-stained smears and expressed as percentages. A combined percentage of aggregate and ring-form reticulocytes was also calculated.

Comparisons were made between adjusted polychromatophil percentage and percentages of each of the 4 reticulocyte types and also the combined percentage of aggregate and ring-form reticulocytes. Comparisons were also made between the combined percentage of polychromatophils and IECs and percentages of the 4 reticulocyte types and also the combined percentage of aggregate and ring-form reticulocytes. To evaluate the potential for observer bias, the differential counts on 1,000 cells were repeated on all NMB-stained smears by both observers at the conclusion of the study.

Table 1—Spearman’s rank correlation coefficient (ρ) value (P value in parentheses) for comparisons of percentages of reticulocytes in each of 5 reticulocyte categories with the adjusted polychromatophil percentage* and with the combined percentage of polychromatophils and IECs determined in blood smears from 31 birds.

*Calculated by exclusion of IECs from counts. †Four types of reticulocytes were defined on the basis of the reticulum pattern revealed via supravital NMB staining as follows: punctate-1 (< 10 dots of stained reticulum); punctate-2 (> 10 dots of stained reticulum); aggregate (any aggregates of stained reticulum, distributed diffusely within the cytoplasm); and ring-form (aggregates of stained reticulum that formed a ring—contiguous or discontinuous—around at least half of the reticulocyte nucleus). A value of P < 0.05 was considered significant for the correlation coefficient.
Correlation of polychromatophil and reticulocyte percentages—On the basis of data collected from examination of Wright-Giemsa- and NMB-stained blood smears from 31 birds by the 2 observers, inter- and intraobserver variations were first calculated for ring-form reticulocyte types. Calculated F (test statistic for ratio of mean square numbers) values were low, and P values were > 0.05 for all comparisons (for interobserver variation, F = 0.072 and P = 0.790; for intraobserver variation for observer 1, F = 0.404 and P = 0.530; and for intraobserver variation for observer 2, F = 0.083 and P = 0.775). Because these results indicated a lack of significant interobserver or intraobserver variation, mean differential percentages for the 2 observers were calculated and used for further comparisons.

Ten comparisons were assessed by use of Spearman rank correlation coefficient (Table 1). Correlation between adjusted polychromatophil percentage and the percentage of ring-form reticulocytes was strongest (r = 0.978; P < 0.001). Correlation between the combined percentage of polychromatophils and IECs and the percentage of ring-form reticulocytes was only slightly less strong (r = 0.976; P < 0.001). These 2 comparisons were plotted, and regression line equations were obtained (Figure 3). Correlation coefficients were substantially lower for the other 8 comparisons. The Deming regression model was used to compare data generated separately by each observer for polychromatophil percentage with data generated for ring-form reticulocyte percentage, and CVs were calculated for both measurement techniques. The CV for polychromatophil quantitation was 19.44%, whereas the CV for ring-form reticulocyte quantitation was 8.43%. The 95% confidence intervals were –0.017 to 0.003 for regression line intercept and 0.910 to 1.160 for regression line slope.

Generation of a reference interval for polychromatophils in healthy psittacines—On review of clinic records, 44 birds were defined as healthy adult psittacines according to the defined criteria. Differential counts on 1,000 RBCs were performed on archived smears of blood from these birds. Data for both the percentage and absolute number of polychromatophils were normally distributed, and no outliers were identified; thus, the reference interval for each was defined as the mean ± 2 SD value. The reference interval for percentage polychromatophils was 0.60% to 8.00% (mean, 3.66%). The reference interval for absolute polychromatophil count was 5,668 to 227,643 cells/μL (mean, 131,204 cells/μL).

Discussion

In the present study, a method of avian reticulocyte counting that was accurately reproduced by different observers was investigated. The 4 categories of reticulocyte morphologic types were representative of classification schemes used by previous investigators. In our study, these criteria were more clearly defined, and in routinely stained blood smears, reticulocyte classifications were correlated with percentage of polychromatophils. For the 2 observers involved in the study, intraobserver CV for ring-form reticulocytes was low (5.61%), which suggested that counting this specific type of reticulocyte may be associated with a greater degree of reproducibility than that associated with counting any of the other 3 reticulocyte types. The results of our comparisons further indicated that the ring-form reticulocyte percentage correlated strongly...
with the polychromatophil percentage. Counting of
ring-form reticulocytes is therefore recommended as
the best method for avian reticulocyte quantification,
given both the strong correlation between ring-form
reticulocytes and polychromatophils and the higher de-
gree of precision in counting ring forms versus other
types of reticulocytes.

A separate category of IECs was created to include
erythroid cells with a morphologic appearance that was
more immature than that of polychromatophils; these
cells are considered analogous to nucleated RBCs in
mammalian blood. On the basis of findings in supravi-
tally stained avian bone marrow,7 the staining pattern of
IECs should be similar to that of ring-form reticulocytes,
and we considered the potential impact of inclusion or
exclusion of these cells in our study via comparisons
with an adjusted polychromatophil percentage (with
exclusion of IECs) and with the combined percentage
of polychromatophils and IECs. Both polychromatoph-
ophil categories quantitatively correlated strongly with
ring-form reticulocytes, with only a slight decrease in
correlation strength when IECs were included. Imma-
ture erythroid cells comprised 0.0% to 5.0% of all RBCs
in the samples included in our study, which was a wide
range of values. The practical implication of this find-
ing is that inclusion or exclusion of IECs in a polychro-
matophil count appears to have a minimal, and likely
clinically unimportant, effect on the percentage of ring-
form reticulocytes.

In addition to percentages of each reticulocyte
type, a combined percentage of aggregate and ring-
form reticulocytes was also compared with the per-
centage of polychromatophils. This fifth category was
added because ring-form reticulocytes arguably repres-
ent a subgroup of aggregate reticulocytes, such that
a category of what might be considered all aggregate
reticulocytes could show the strongest correlation with
polychromatophils in avian species, as is true in do-

cestic cats.11,12 However, strong correlation between
the percentage of polychromatophils and the combined
percentage of aggregate and ring-form reticulocytes
was not detected in our study.

Lack of uniformity in counting and reporting of
avian reticulocytes has been identified as a likely cause
of variation in published reticulocyte counts in chick-
ens.10 The data from the present study supported this
theory. In the 1950s and 1960s, reported values for
reticulocytes in clinically normal humans also varied
markedly, and several authors attributed this to a lack
of uniform criteria for identification of reticulocytes.13
Standardization of a uniform reticulocyte-counting
technique should reduce variation in proposed refer-
ence intervals in avian species, as has been the case for
mammalian species.

Supravital staining of avian bone marrow has re-
vealed the presence of a perinuclear ring of reticulum in
cells in the early stages of erythroid cell development.7
Avian erythrocytes do not extrude their nucleus during
differentiation as do mammalian erythrocytes, but a ma-
turation stage similar to that of a late metarubricyte
was believed to be indicated by dissolution of the perinu-
clear ring of reticulum into a diffuse distribution of granu-
lar reticulum throughout the cytoplasm.7 The results of
our study suggest that this transformation from a peri-
nuclear to a diffuse distribution of reticulum is more
likely to be analogous to the maturation of a polychro-
matophil to a mature erythrocyte because the percent-
age of ring-form reticulocytes (ie, those with perinu-
clear granulation) quantitatively correlated much more
strongly with the percentage of polychromatophils than
did the percentage of aggregate reticulocytes (ie, those
with diffusely distributed granulation) or the combined
percentage of aggregate and ring-form reticulocytes.

In the present study, the optimal avian reticulo-
cyte staining protocol involved a 1:3 dilution of whole
blood to NMB stain with an incubation time of 10 min-
utes. Ratios of whole blood to supravital stain ranging
from 1:1 to 1:3 have been used previously for staining
of blood from both avian and mammalian species.6,14
Both the type and concentration of the supravital stain
used must be taken into account when comparing stain-
ing methods. The 1:3 dilution was selected as optimal
in our study because cytoplasmic staining was better
defined at this dilution than at lower dilutions.

Refrigerated storage of whole blood for as long as
24 hours minimally affected reticulocyte staining and
counting in our study, although there was a low per-
centage (0.6%) of erythrocytes that were degenerated in
appearance and could not be included in the differential
count. After 48 hours of refrigerated storage, many cells
were degenerated and deeply stained, and reticulocyte
counting could not be performed. Therefore, our rec-

commendation is to store avian whole blood in a refrig-

erator for no more than 24 hours prior to reticulocyte

counting and counting.

Hematology reference intervals for healthy birds
can be difficult to establish, in part because of popula-
tion sample size and sample volume limitations, and in
part because of the wide phylogenetic diversity of avian
patients. Because of the strong correlation between per-
centages of polychromatophils and ring-form reticu-
locytes detected in the present study, which included
blood samples from birds in 4 different orders, we pro-

posed that a polychromatophil reference interval could
be used as a substitute for a reticulocyte (ring-form)
reference interval. The ability to assess erythrocyte re-
generation from examination of routinely stained blood
smears would save time and avoid the need for collec-
tion of an additional sample volume for a reticulocyte
count. The polychromatophil percentage reference in-

terval generated in our study (0.60% to 8.00%) was con-
sistent with published estimates13 of polychromasia in
healthy birds. An absolute polychromatophil reference
interval was also generated, but no published estimates
of this interval could be found for comparison. Because
of the proportionate bias between percentages of poly-

chromatophils and ring-form reticulocytes, the poly-

chromatophil reference interval should be used only as
an estimate of, rather than as a replacement for, a refer-

ence interval for ring-form reticulocytes. In addition,
interobserver CV in the present study was considerably
higher for counts of polychromatophils than it was for
counts of ring-form reticulocytes (19.44% and 8.43%,
respectively), which indicated that there was a greater
degree of random error, and therefore greater impreci-
sion, associated with counting polychromatophils than
counting ring-form reticulocytes. Observer detection of polychromatophilia is based on color alone and is therefore likely to be a more difficult and subjective assessment than detection of reticulocytes. Decreased interobserver agreement in blood smear evaluation has been correlated with color vision deficiencies, and a pattern-based system rather than a color-based system is clearly advantageous to a practitioner with color vision impairment. For these reasons, reticulocyte counting is the preferred laboratory method for evaluation of regenerative erythrocyte responses in birds, and reference intervals for ring-form reticulocytes should be developed by laboratories in which avian reticulocyte counts are performed.

a. Euthasol, Virbac Inc, Fort Worth, Tex.
b. 0.5% new methylene blue stain, Ricca Chemical Co, Arlington, Tex.
c. 7100 Aerospray stainer, Wescor Inc, Logan, Utah.
d. ADVIA 120, Bayer Diagnostics Division, Bayer Corp, Tarrytown, NY.
e. MedCalc for Windows, version 8.1.1, MedCalc Software, Mariakerke, Belgium.
f. SPSS for Windows, version 11.5, SPSS Inc, Chicago, Ill.

References