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Stress Assessment by the Hemogram Method - Circulating Cells Complicating Reliance on Heterophil/Lymphocyte (H/L) Ratio

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Abstract

The subject is a reexamination of the utility of the heterophil/lymphocyte ratio as a stress measure. The data are obtained from blood films of non-experimental chickens at 3 weeks of age and housed in isolator units. Standard differential counts of 2 x 200 cells indicated total white blood counts (TWBC) were in the range of 30-200(K) with an average of ~100(K); normal to leukemoid reaction levels (N= 23 samples). The H/L average of ~ 0.5 was typical of a non-stress hemogram. However, many atypical cells were identified including small lymphocytes with irregular cell membranes (zeiosis) reactive lymphocytes, resting (small) or activated NK (natural killer lymphocytes) unusual heterophils of three types: classic (HC), typical (HT), variant (HV), and early stages of the granulocyte series. Aggregates of atypical cells (reactive clusters) were also common. Atypia were present in blood samples at all TWBC and H/L levels. These hematological conditions suggest that estimates of stress status solely reliant on H/L data may not convey an accurate blood picture. It is necessary to integrate the calculated H/L with the TWBC and the occurrence of atypical cells to more accurately determine homeostasis.

ABBREVIATIONS

H: heterophil (HC, classic, HV, variant, HT, typical) Ls: small lymphocyte ~6 µm diameter, Lm: medium lymphocyte, Mn: monocyte, Ba: basophil, Eo: eosinophil, TWBC (K): total white blood cells per cubic mL in thousands (K), H/L 1 = (HC + HT + HV) / Ls; H/L 2 = (HC + HT + HV) / (Ls + Lm); ΔH/L= H/L1 – H/L2, cm: cell membrane, A: area µm^2

INTRODUCTION

The heterophil/lymphocyte ratio (H/L) is widely used as a technique to estimate stress [1]. Its basis is the principle that stress alters homeostasis by affecting the adrenal-corticoid axis. High glucocorticoid levels change the blood profile causing leukopenia (lymphocyte) and leukocytosis (heterophil); the H/L is raised as a consequence [2]. The data are obtained by direct hemacytometer counts, or extracted from standard differential counts (SDC) of whole blood. Automated procedures sometimes replace manual methods.

However, a number of difficulties associated with interpretation of the H/L derive from its computation, and others from the existence of cytological atypia. Few investigators describe the exact computation method used for their H/L value [3]. Should reactive lymphocytes enter the denominator? How are the several distinct heterophil types considered in determining the numerator? How do atypical cells affect the interpretation of the hemogram? [3] Is cell size considered? [4,5]. These and other questions remain unresolved. Collectively these difficulties challenge the utility of the H/L as a simple means to evaluate stress or test theories.

The purpose here is to describe cells having the potential to complicate interpretation of the avian hemogram and the derivative statistic, H/L. The focus is on cells directly entering the H/L computation, either as components of the numerator, or the denominator. An additional purpose is to illustrate examples of atypical cells, themselves an indication of a complex hemogram. Equivalents of these atypical cells are likely to occur in a broad range of species and so be of interest to a wide array of investigators.

MATERIALS AND METHODS

Animals

Chicks were obtained from an SPF flock certified free of 33 avian pathogens, Sunrise Farms, Catskill, NY 12414, USA. They were housed in Horsfall-type negative-pressure isolators. The chicks were given food, free of anticoccidials, and water *ad libitum*. They were examined daily and determined to be clinically healthy and sero-negative to all known chicken diseases. Wing-vein blood was drawn at 3 weeks of age, prior to the use of these animals in experiments. Additional management details are in Cotter and Heller.[6]

Stain Procedures

Monolayer films made by spreading approximately 3 µL

of blood across alcohol cleaned glass microscope slides were air-dried and immersed in 100% MeOH. Films were stained by using an in-house version of Wright's method followed by a brief secondary exposure to Giemsa (Sigma).

Standard Differential Count

Two counts of 200 leukocytes/slide were sorted using criteria as described by Lucas and Jamroz[7] and Cotter [3,6,8]. The designation "typical heterophil" (HT) as used here was assigned to the most frequent type seen in earlier studies. Classic heterophils (HC) resemble those most often illustrated in avian hematology literature. Rare variant heterophils (HV) are distinct from both HT and HV [6,8,9]. Total white blood counts (TWBC) were determined by a modified microscopic method as described in Campbell [10]. Standard Differential Count (SDC) was determined at 40x magnification.

H/L Ratio Calculation

Division of the sum of all three heterophil types by the small "resting" lymphocytes (Ls) gives the H/L 1; [H/L 1 = (HC + HT + HV) / Ls]. Division of the same heterophil value by the sum of all lymphocyte types, (resting Ls, medium reactive (Lm) gives the H/L 2; [H/L 2 = [HC + HT + HV) /(Ls + Lm)]. ΔH/L = H/L 1 − H/L 2.

Light Microscopy and Photomicrographs

Olympus CX-41(Olympus America, Center Valley, PA 18034-0610) equipped with Plan N 40x, 0.65 numerical aperture dry, and Plan N, 1.25 numerical aperture 100x oil objectives. All images were captured at 40x or 100x with an Infinity-2 1.4-megapixel charge-coupled device Universal Serial Bus 2.0 Camera, and processed with Infinity Analyze software (Release 5.0.3) (Lumenera, Inc., Ottawa, ON, Canada).

Graphics

Graphics were produced with Minitab Statistical Software (Release 17 for Windows, State College, PA).]

RESULTS

The SDC (%) for the samples described here is given in Table 1 along with the average for the entire flock. The scatter plot distribution for H/L 1 and H/L 2 pairs for the corresponding text figures are in Figure 1.

Table 1 indicates the TWBC for 3 samples providing the photographs were in the leukocytosis (> 50K) to leukemoid reaction (>100k) range. The corresponding H/L values were either low (samples 1,2) or in the non-stress range (samples 3,6) or stress (4,7; Table 1).

As indicated by Figure 1 the samples providing the photographs came from SDC's distributed across the range of H/L values. In 3 cases (samples 3,6,7; Figure 1) duplicate SDC's results were distinct as indicated by the separation of data points. This is caused by sorting, the non-uniform distribution of cells in reactive samples (Figure 2).

CytologResting Lymphocytes

A mixed field displaying atypia of several series is in Figure 2. Ls* are small "resting" lymphocytes with irregular cm (zeiosis, arrows). HC* is a classic heterophil with weakly stained nucleus and defective (undifferentiated) cytoplasmic granules. Medium reactive lymphocytes (Lm*) and atypical thrombocytes are present; N is the remnants of a lysed nucleus (likely a RBC).

Reactive Lymphocytes

Reactive lymphocytes (Lm, Figure 3, Panel A) are rarely seen in normal hemograms. These moderately sized cells (Lm 1, A 50 μm^2 and Lm 2, A 36 μm^2) are noticeably larger than the nearby resting lymphocyte (Ls, A 12 μm^2). The lower Lm N/C ratios (~ 0.7) contrast with the Ls (N/C ~ 0.9) and so these are likely developmental plasmacytes. When granulated lymphocytes (NK cells; Panels B and C, Figure 3) appear in a hemogram a further difficulty arises. These cells can indicate an active (anti-viral) immune response is already in progress [11]. Furthermore,

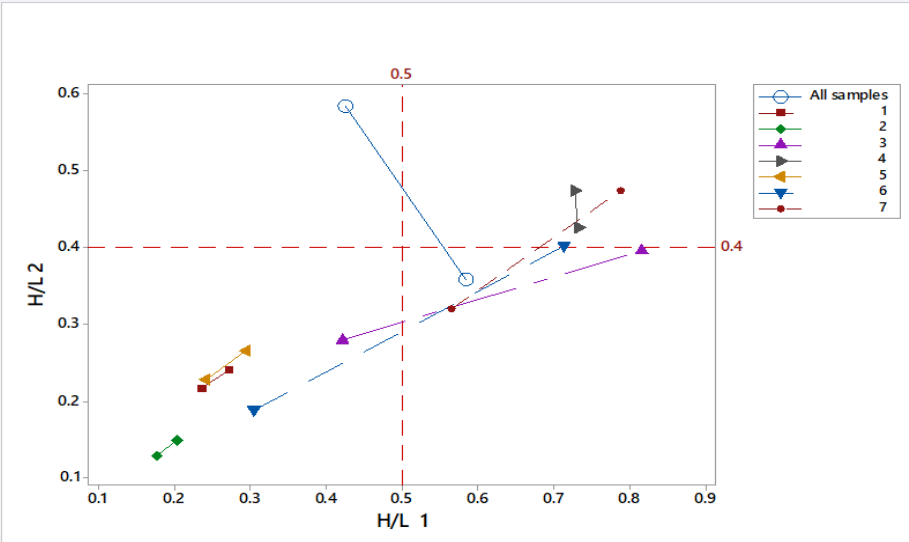

Figure 1 Paired scatter plot of H/L 2 vs. H/L 2 for the 7 slides providing figures, and all 23 samples (open circles) from an isolator housed 3 wk SPF flock. Reference lines indicate assumed non-stress cut-off values; H/L 1, 0.5; H/L 2, 0.4.

Table 1: Average of differential counts (2 x 200 cells) as a percent of TWBC and heterophil/lymphocyte ratios for text figures. H/L ratios were determined from 2 SDC counts starting at 5 and 10 mm of the microscope stage and continued until at least 200 cells were sorted. TWBC were estimated from the SDC slides.

Sample	HT	HV	HC	Ls	Lm	NK	Bst	Mn	Ba	Eo	H/L 1	H/L 2	ΔH/L	TWBC(K)
1	8.8	0.9	7.2	66.7	7.2	0.0	0.7	1.6	6.9	0.0	0.25	0.23	0.02	40
2	4.1	1.1	5.3	55.6	20.4	0.0	0.0	0.7	11.9	0.9	0.19	0.14	0.05	50
3	9.6	1.4	12.0	40.0	29.0	0.0	0.2	0.5	7.2	0.0	0.62	0.34	0.28	50
4	26.5	1.9	1.7	41.4	25.8	0.0	0.5	0.2	1.9	0.0	0.73	0.45	0.28	200
5	11.4	2.1	3.0	62.3	5.1	4.7	0.0	7.7	3.7	0.0	0.27	0.25	0.02	100
6	18.2	2.9	0.0	43.5	29.9	0.0	0.0	2.9	2.6	0.0	0.51	0.29	0.21	140
7	25.2	2.0	0.0	40.3	28.9	0.0	0.2	0.0	3.4	0.0	0.68	0.40	0.28	200
All	14.8	1.9	4.5	47.3	20.3	0.4	0.1	4.6	6.1	0.0	0.50	0.32	0.18	107

Abbreviations: H, heterophil (HC, classic, HV, variant, HT, typical) Ls small lymphocyte ~6 µm diameter, Lm medium, large (diameter 8–10 µm) NK, natural killer, Bst, granulocyte blast, Mn, monocyte including, Ba, basophil, Eo, eosinophil. TWBC (K), total white blood cells per cubic mL in thousands (K). H/L 1 = (HC + HT + HV) / Ls; H/L 2 = (HC + HT + HV) / (Ls + Lm); ΔH/L= H/L1 – H/L2.

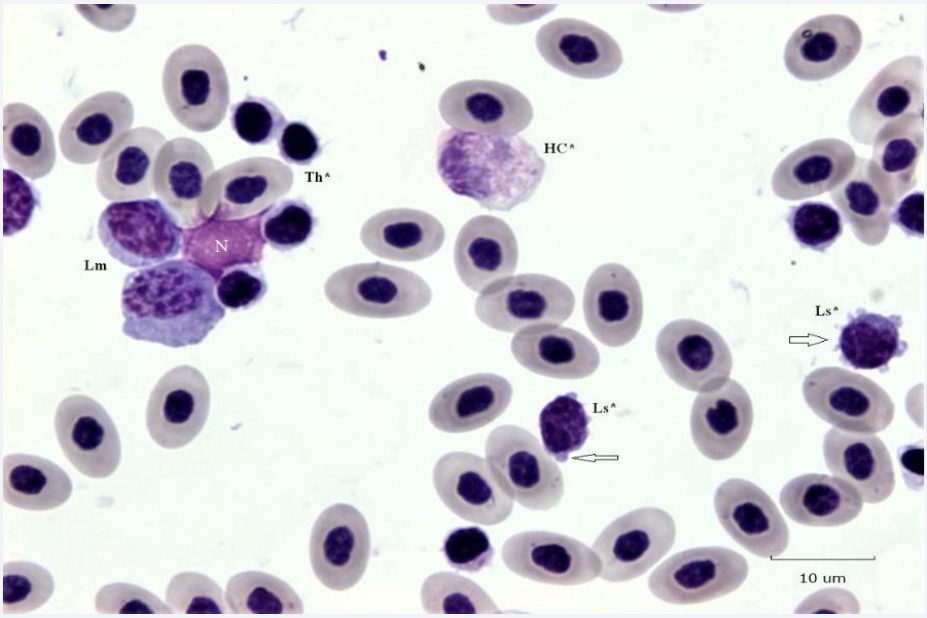

Figure 2 A mixed field with atypia of several series. Ls* small "resting" lymphocytes with irregular cm, zeiosis (arrows). HC* classic heterophil with weakly stained nucleus and defective (undifferentiated) cytoplasmic granules. Medium reactive lymphocytes (Lm*) and atypical thrombocytes; N is the remnants of a lysed nucleus (?RBC). Collectively the Lm/Th/N/RBC aggregate is a "reactive cluster". Additional descriptions of cells are in the text.

developmental cells of the granulocyte series, themselves an indication of inflammation, can be mistaken for NK cells (Figure 3, Panel D).

Heterophil Granulocytes

Traditionally placement of avian granulocytes is into one of three groups, heterophil, basophil, or eosinophil [7,10]. The cytoplasmic granules of chicken heterophils and eosinophils are red but shaped differently. The fusiform (spindle) shape of heterophil granules aids their differentiation from eosinophils whose granules are spherical. Granule stain intensity, the central bodies of classic heterophils (HC) and variation of nuclear configuration are additional distinctions among heterophil types. Basophils are also granulocytes whose deep purple metachromatic spheres allow easy differentiation from other granulocytes. All granulocytes develop from a common stem cell, but mature cells descend from distinct metamyelocyte progenitors [7]. Heterophils are not a single series. Three distinct types (typical, HT; classic HC, and variant, HV) differentiated by granulation and nuclear configuration have been described [5,6,9]. HT types, often the most frequent, bear some resemblance to mammalian neutrophils (Figure 4).

Further examples of HV and HC differentiation are given in Figure 5. Panel A. Standard sized classic heterophil (HC, A 58 μm^2) is compared with medium sized variant types (HV 1 and 2; A ~ 48 μm^2) and atypical small lymphocytes Ls (1, 3). The HC granules are poorly stained in contrast with those of the HV. This indicates an HC granulation defect rather than a staining artifact. A true "resting" Ls (2) N/C ~1, is also in the same field. Panel B. A

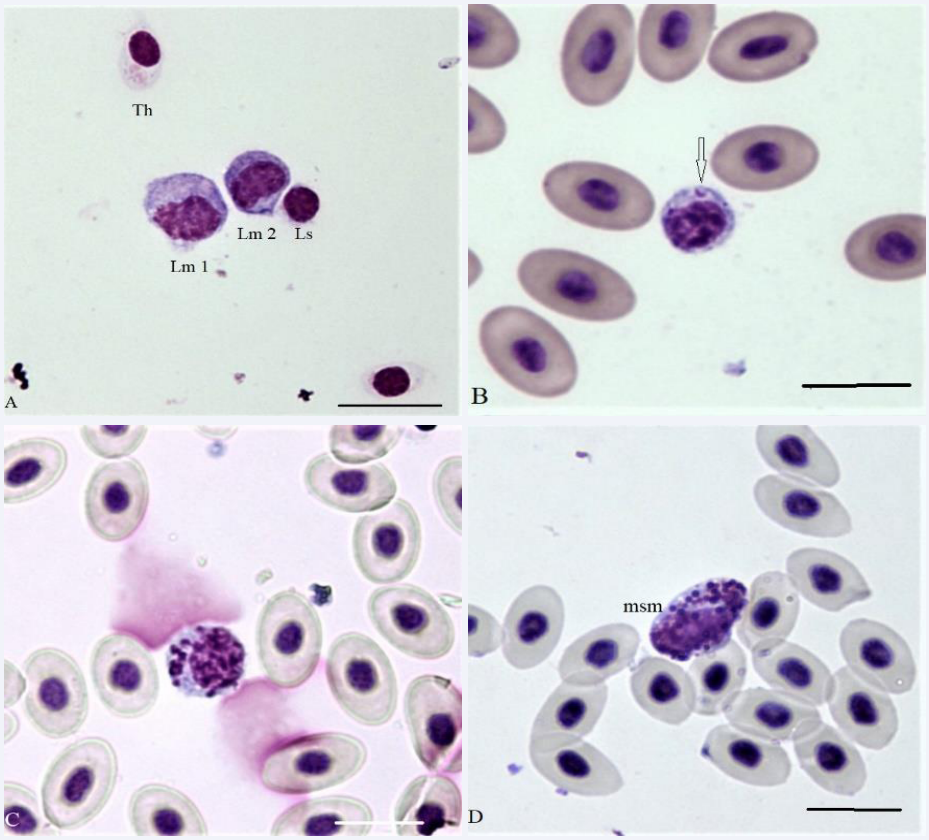

Figure 3 Panel A. Reactive lymphocytes (Lm) are differentiated form small resting lymphocytes (Ls). Panels B-D. NK (natural killer) lymphocytes contain cytoplasmic granules (arrow Panel B) and can resemble cells of the granulocytic series (mesomyelocyte, msm, Panel D). Bar 10µm. Additional descriptions of cells are in the text.

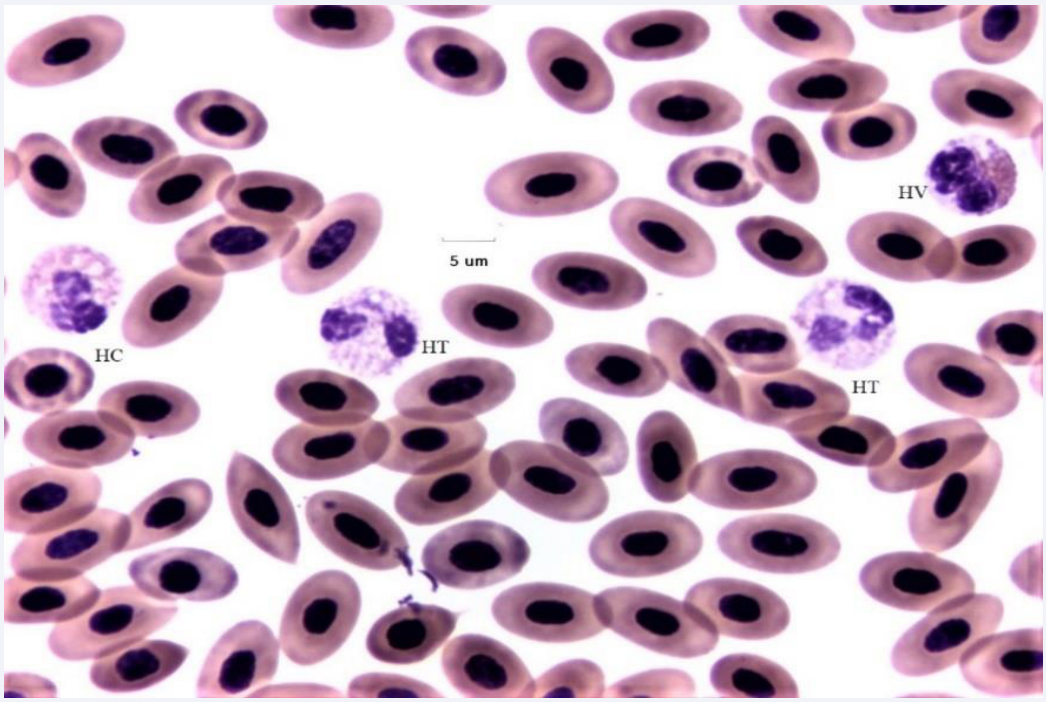

Figure 4 Examples of standard sized (R ~5 µm; A ~ 80 µm2) classic (HC) typical (HT) and variant heterophils (HV) from a 6 wk SPF chick. Classification is based on cytoplasmic granulation and nuclear configuration differences. Additional descriptions of cells are in the text.

large HV (A 85 μm^2) and a medium lymphocyte are in transition to the reactive state (Ls/Lm, A 55 μm^2). The cytoplasmic granules of the HV are orange spheres, distinct form the red spheres of eosinophils, and are often restricted to one side of the nucleus. The HV in panel B has fewer cytoplasmic granules than are ordinarily found. Thrombocyte (Th) shape and size irregularities, indicating they are reactive, are seen in both panels (Figure 5,6).

EOSINOPHILS

Eosinophils (Figure 7) are sometimes mistaken for classic heterophils. A late eosinophilic metamyelocyte (mtm) has a non-segmented nucleus with coarsely condensed chromatin and red spherical cytoplasmic granules (A 38 μm^2); a necrotic heterophil (HC or HT) is at the top right. Panels B, C, and D contain additional examples of mature (2 nuclear lobes; A ~39 μm^2) Eo. A faint pseudopod projects from the Eo of panel D (arrow). Eosinophils are differentiated from HV type heterophils by their red cytoplasmic granules and smaller sizes (Figure 7).

DISCUSSION

The objective of this manuscript is to describe circumstances where determination of stress by the H/L method is not a straight forward process. This arises because often no careful description of the method used to calculate the H/L is provided. This deficiency can render data comparisons between studies and tests of theories problematic. No statistical transformation technique (see Valdebenito [12] for an example) can overcome such difficulties.

Furthermore, the inclusion of reactive cells is rarely declared. Nor are cut-off values separating stress from non-stress firmly established. Recognition of atypia is important. Toxic and apoptotic heterophils are potentially injurious and thus should not be found in a normal (non-stress) hemogram [13].

Small "resting" lymphocytes can be problematic if they are atypical. The Ls* of Figure 1 are an example of cells whose surrounding membranes have developed projections (blebs)

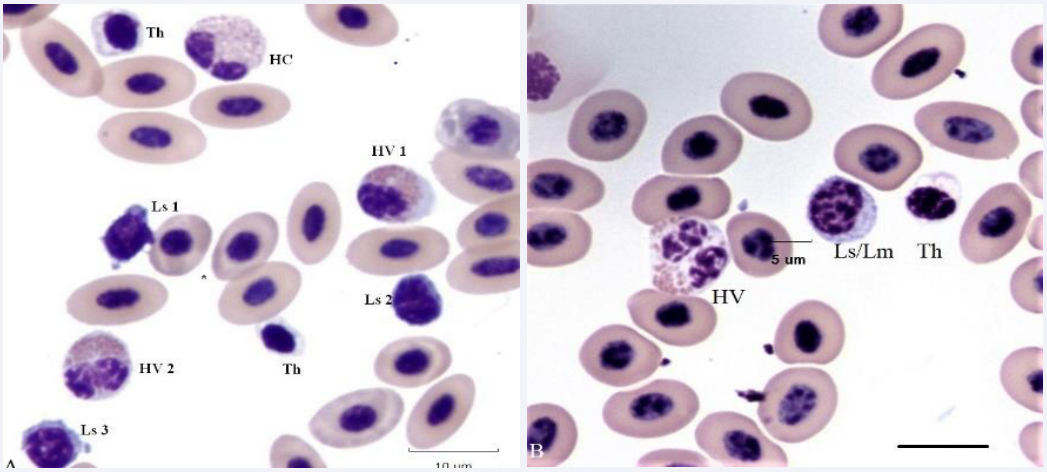

Figure 5 Further examples of variant heterophils (HV) and reactive/atypical lymphocytes. Panel A. Standard sized classic heterophil compared with medium sized and atypical (zeiosis) Ls (1, 3). Panel B. Large HV (A 85 μm2) and medium lymphocyte in transition to the reactive state (Ls/Lm, A 55 μm2). Thrombocytes (Th) are in both panels. Bar 10 μm.

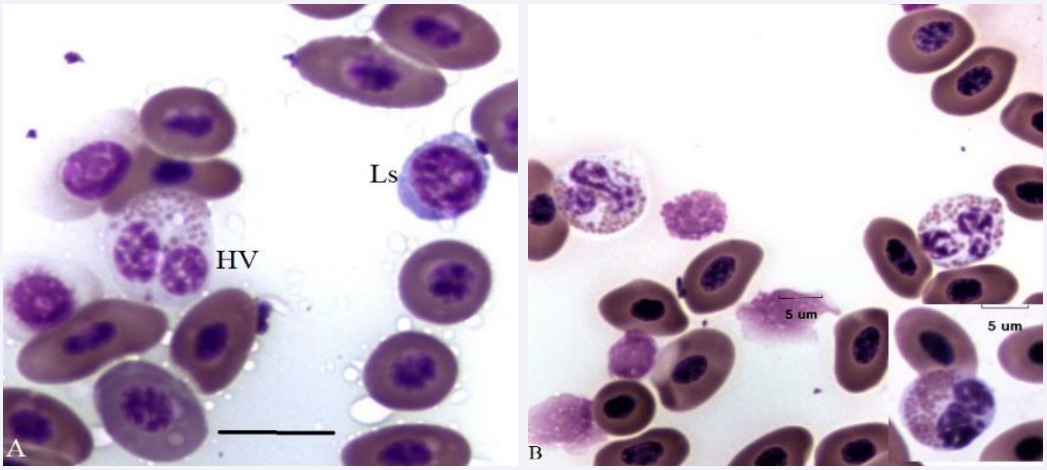

Figure 6 Granule number deficiency in heterophils. Examples of standard sized (Ave. area 88 μm2) variant heterophils (HV) with apparent deficiency of cytoplasmic granules. An HV with a full complement of cytoplasmic granules is in the inset of panel B.

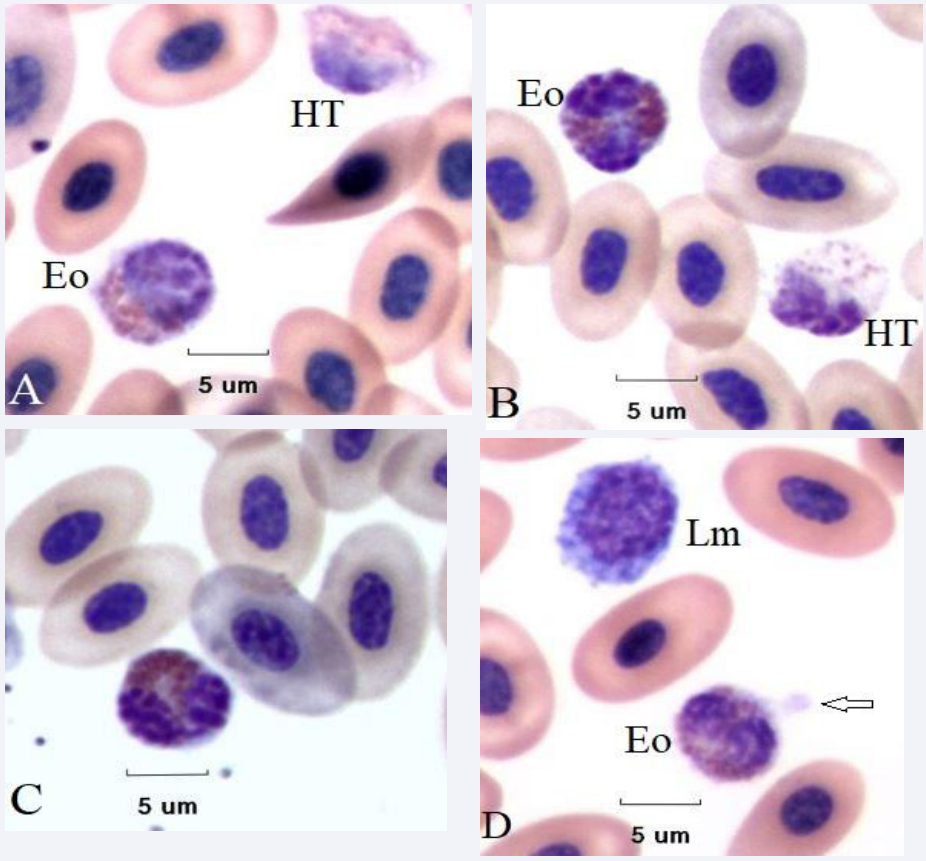

Figure 7 Panel A. A late eosinophilic metamyelocyte (mtm) chromatin and red spherical cytoplasmic granules (A 38 µm2); a necrotic heterophil (HC or HT) is at the top right. Panels B, C, and D contain additional examples of mature (2 nuclear lobes; A ~39 µm2) Eo. Additional descriptions of cells are in the text.

often considered as zeiosis, a prodromal stage of apoptosis (programmed death).

Heterophils, the H of the H/L are diverse, differing in granulation and nuclear condition, justifying the separate categories (HC, HT, and HV). Moreover, toxic and other forms of atypia as giant cells, and dwarfs, can occur among any H type adding another level of complication [4].

The present study is limited because physiologic differences of granules among heterophils types have not yet been established. It may be that either HT or HV are capable of myeloperoxidase production, a property not in HC [14] Furthermore, the differential phagocytic capacity of each heterophil type is not known, nor is type-specific toxicity differences of necrotic heterophils. However, recognition of subtle toxic changes has been ignored (see Figure 1 of Davis [1] for an example).

Recently an apparent novel type of apoptosis occurring in HC types has been described [8]. Whether this process is restricted to the HC type or can occur in HT and HV is not yet known. It may account for some H/L variation if HC types are underrepresented in the SDC; because of lysis an artificially low value could result.

NK cells were found in a minority 3/23 (13%) of samples and were between 1 and 5% of the SDC. Some NK with only a few cytoplasmic granules cells were probably the resting type; others with more granules are probably the reactive type suggesting viral infection [15].

In summary, some weaknesses of the H/L method earlier described have been extended by the present observations [3,5]. These include how the H/L is actually calculated and the effect of atypical cells [9].

CONCLUSION

The data presented here expand upon an earlier study examining the utility of the H/L ratio as a stress measure [3]. Atypical cells of that study were seen in blood films from hens in several types of commercial cages. Those hens were sampled between 18 and 77 wk. Here younger chickens (3 wk) housed in isolators, and free of known disease, are the subject. Interestingly the isolator samples contained many atypical and reactive cells. NK cells, for example, are indicators of viral infection; atypical heterophils are common in blood also containing bacteria [9]. It is likely that in the present study microbial contaminants of blood came from the gut. The results show that in the presence of atypia, as described here, the use of a simple H/L ratio fails to establish stress status.

These data are intended to draw attention to the need for consensus among investigators who use the H/L method in their choice of cells included in its computation. Atypical or reactive

lymphocytes should not be included in the denominator. The choice of heterophils is also important. As it is not currently known if HT and HV types are physiologically distinct from the better-known HC; either may indicate a complex hemogram. Moreover, low H/L ratios in samples with high TWBC cannot be interpreted as non-stress. The question of leukopenia has not been adequately addressed. Finally, caution must be given to samples with atypical cells of the other series not directly used for the H/L.

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