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A Microscopic Study on Morphology of Reactive Thrombocytes in Duckling

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ABSTRACT

Thrombocytes, well known as important clotting factors, and now known to be important as phagocytic cells, might benefit the study of the avian hemogram. Therefore, blood was sampled from 4 late-stage embryos at embryo day 24, and 4 one-day-old hatchlings (d1), and 5 female ducks aged 59 weeks stained by Wright-Giemsa and examined at 100x. Standard differential counts (SDC) of 2 x 200 cells were used to determine total white blood counts (TWBC) and heterophil/lymphocyte (H/L) ratios**.** Thrombocytes were not included in the SDC but were studied from photomicrographs. Reactive thrombocytes were present in blood films having a normal TWBC or in the presence of leukocytosis (59 weeks). The H/L ratios may or may not be elevated. Reactive thrombocytes can be differentiated from quiescent types on morphologic criteria. These included an increase in the number of magenta "specific granules", the development of cytoplasmic vacuoles, and a capacity to form aggregates with other Th or with cells of another series. Reactive Th were not necessarily larger in size than quiescent types. In some instances, Th aggregation with RBC (toroid formation) was with sufficient force to distort the RBC cell membrane. It was observed that reactive thrombocytes were accompanied by bacteria, either free-swimming or attached to cell-associated bacteria. Reactive thrombocytes having lost portions of their cell membrane were regularly encountered. As avian thrombocytes are now recognized as important phagocytic cells, as well as having a primary role in hemostasis, they are part of the immune defense mechanism. The presence of reactive thrombocytes in a hemogram should be considered when using hematological data to evaluate immune responses and establish stress status.

Keywords: Hematology, Immunity, Reactive thrombocyte, Simple and complex toroid, Stress

INTRODUCTION

Avian thrombocytes (Th) appear to parallel the prominent role in hemostasis played by mammalian platelets (Ferdous and Scott, 2015). They initiate clotting, repair damaged endothelium, and accumulate at the site of injury where they block blood leakage (King, 1980). One study suggested they are less important in (early) clotting due to a deficiency of thromboplastin (Handlinger, 1989). Morphological changes in thrombocytes associated with stress were described by Gross (1989) who classified thrombocytes with a system of scores.

In normal circulation, thrombocytes are more numerous than other leukocytes occurring at approximately 30K/µL in chickens (Lucas and Jamroz, 1961). Thrombocyte phagocytosis was demonstrated microscopically using a Trypan Blue dye particle uptake assay (Carlson et al., 1968). *In vitro* studies demonstrated duck thrombocytes change from oval shapes to spheres after exposure to adenosine diphosphate (ADP), and they are aggregation by shaking (Grant et al., 1973). Thus, a change of shape can also be a normal physiological response of thrombocytes and may account for some of their morphological variations (Lucas and Jamroz, 1961).

The phagocytic capacity of thrombocytes for several types of bacteria was investigated by Wigley et al. (1999) who showed activity against *Salmonella* and other Gram-negative species. Ferdous et al. (2008) indicated Interleukin-12 (IL-12) is produced by thrombocytes exposed to lipopolysaccharide (LPS). Toll-like receptor production by reactive thrombocytes accompanies the release of cytokines from their storage granules, suggesting thrombocytes respond to viruses as well as bacteria (St Paul et al., 2012). Collectively, these observations indicate the dynamic nature of the

thrombocytes. They participate in a variety of immune functions beyond their hemostatic duties. A recent review of avian thrombocyte biology, including observations on duck thrombocytes, outlining their morphological and immunological properties is reported by Astill et al. (2022). With this in mind, the purpose of the current study was to illustrate various cytological properties of thrombocytes as they transition from a quiescent to a reactive state.

MATERIALS AND METHODS

These observations came from blood sampled from commercial stocks not otherwise manipulated; or from older breeding stock ducks.

Late-stage embryos and day of hatch samples

The data were obtained from light microscopic observations of thrombocyte behavior in duck peripheral blood. Shape change, specific granule elaboration, vacuole development, phagocytosis, and adherence to other thrombocytes and leukocytes were illustrated. All were observed in late-stage embryos at embryo day 24 (Ed24), hatchlings at day 1 (d1), and 5 breeding ducks aged 59 weeks (59 weeks). Thrombocytes were often found in the presence of either free-swimming or cell-associated bacteria (CAB). Eight blood smears prepared at a hatchery were sent to Cotter Laboratory, Arlington, MA, USA, for the current study. The samples of jugular vein blood came from 4 Ed 24 and 4 d1 hatchlings. They were post-fixed by immersion in 95% EtOH for 15 minutes followed by staining (6 minutes) using an of Wright-Giemsa method (Cotter, 2021b).

Breeding ducks

Blood samples taken at 59 weeks came from a Berne, Indiana, USA flock chosen because of a history of poor fertility. Whole blood (1-3 mL) drawn from a leg vein was placed into EDTA tubes. To avoid the storage effects, monolayer films were made within 24 hours of collection. Approximately, 3 µL of blood was spread across the length of alcohol-cleaned glass microscope slides and dried immediately with a hot air stream. Slides were immersed in 95% ethanol and postfixed for 10-15 minutes. Staining was done as described above.

Welfare

Ducking welfare was monitored under the Maple Leaf Farms Trident Stewardship Program for Duck Well Being with procedures reviewed by a PAACO-certified auditor and licensed Veterinarian.

Light microscopy and photomicrographs

An Olympus CX-41 (Olympus America, Center Valley, PA 18034-0610) was equipped with Plan N 40x, 0.65 numerical aperture dry, and Plan N, 1.25 numerical aperture 100x oil objectives. Images were captured at 100x with an infinity-2, 1.4-megapixel charge-coupled device Universal Serial Bus 2.0 Camera, and processed with infinity analyze software (Release 6.5, Lumenera, Inc., Ottawa, ON, Canada).

RESULTS

The first illustrations of normal (quiescent) thrombocytes were observed in Ed24 and d1. These were followed by examples of cells illustrating changes in cytology from the normal (quiescent) to reactive states. Lastly, examples of reactive thrombocytes (toroids) in 59 weeks ducks were given. The standard differential counts (SDC) for Ed24 and d1 blood films are presented in Table 1. Detailed SDC data for the older ducks were not included. However, the average total white blood counts (TWBC) in 59 weeks flock was 42K with heterophil/lymphocyte$_1$ (H/L1) ~7 and heterophil/lymphocyte$_2$ (H/L2) ~1.8. These values indicate leukocytosis and stress, respectively.

Normal thrombocytes

Thrombocytes not displaying reactivity signs are classified as normal or quiescent cells. They may touch or overlap other cells with which they are not otherwise engaged. (Figure 1A, hatchling at d1). They are oval-shaped and contain 1 or 2 specific granules (magenta) usually located at opposite poles. Cell area (Ac) ~ 20 µm^2; Nuclear/Cytoplasmic Ratio (N/C) ~ 0.4. Generally, they occur as individuals within a field where they may overlap RBCs. Their pellucid cytoplasm appears devoid of hemoglobin. Figure 1B is a quiescent cell (Th1) in a field with CAB and free (encapsulated) bacteria located by asterixis at Ed24. The Th2 share a CAB with an RBC. A lymphocyte (Lp) is a large plasmacytoid cell displaying a paranuclear Hof (Golgi; Cotter, 2022). These examples appear in fields also containing an atypical classic heterophil (HC) and a reactive basophil (Cotter, 2017; Cotter, 2021).

Reactive thrombocytes

During the transition to the reactive state vacuoles develop in the thrombocyte cytoplasm (Figure 2A). The shape can change from an elliptical to a rounded form. This may occur without a noticeable increase in cell area (A_C ~20 μm^2) as illustrated by the paired cells in the lower corner (Figure 2B). The shape change is accompanied by the development of one or two ectactic vacuoles, here occupying only one pole. Vacuole development is sometimes accompanied by an increase in the number of specific granules. The former pellucid cytoplasm also acquires a slightly deeper stain. Surface changes detected microscopically are Th/Th adhesions and attachments to cells of another series. The overall picture of the transition from a normal quiescent field to reactivity is the replacement of space occupied by RBCs or thrombocytes with white blood cells (WBCs). The size of the nucleus in a nearby pRBC suggests it is a tetraploid (4C) cell containing twice the DNA of a diploid (2C) cell. The second example of a thrombocyte with an ectactic vacuole is at the right (Ed24 sample). A nearby RBC has an encapsulated cell associated bacteria (CAB) at its surface, and two non-attached bacteria are at the bottom center.

Adhesion of a thrombocyte to an eosinophil (Th/Eo) is shown in (Figure 3A) where the magenta-specific granules found at each pole appear to be separated from the cytoplasm by a clear space. An encapsulated bacterium has attached to the surface of a normal thrombocytes; at the left. Additional free bacteria located with asterixis and CAB are distributed throughout the field (Figure 3B). An example of phagocytic (located by an arrow) Th/Th attachment in a field where thrombocytes overlap RBC and free bacteria asterixis. The HC is a faintly stained (atypical) classic heterophil containing an intact phagocytosed bacterium. The presence of a capsule and attachment of bacteria to thrombocytes is not accidental but indicates a sub-microscopic change of the thrombocytes cm.

Thrombocyte deterioration was characterized by the loss of cytoplasm and specific granules as illustrated by the appearance of irregular-shaped cells (Figure 4B, Top). An absence of thrombocyte vacuoles was evident. The anuclear remnants of a net-type basophil that have entrapped multiple bacteria are located on the left (Cotter, 2017).

Variation of thrombocytes at Ed24 (Figure 5A). Th1 was normal and intact and has a full complement of cytoplasm, several specific granules, and no vacuole. Th2 was a reactive cell of the pseudopod type, possible equivalents of score 4 and 5 types described by Gross (1989). Th3 was a reactive cell nearly devoid of cytoplasm, N1 and N2 are nuclear remnants of uncertain origin. Background RBCs are normal and fully hemoglobinized but show some degree of overlapping (rouleaux). The variety of reactive and normal thrombocytes in a single field suggests the transition to a reactive state is an individual decision made by each Th, and perhaps under a stimulus initiated at a site remote from the photographic field.

A reactive thrombocyte was in a field in a hatchling (d1) along with an atypical heterophil (HC) and eosinophil (Eo) a reactive plasmacytoid lymphocyte (Lp); CAB (arrow) and free bacteria (circle). Figure 6B. Aggregated thrombocytes in an Ed24 field with free bacteria (asterixis) and CAB (arrows) and a polyploid blast cell (Bst).

The capacity of a thrombocyte to aggregate with another thrombocyte or a cell from another series, called a "toroid". It is illustrated in Figure 7A by a toroid composed of Th-RBC-Th aggregates found in a 59 weeks duck. One member of the toroid called a thromboplastid (Thp) is the anuclear equivalent of an erythroplastid, an anuclear RBC, often seen in duckling bacteremia. It contains an apparently intact phagocytosed bacterium (arrow in figure). Figure 7B is a toroid composed of multiple layers, abbreviated as $(Th\text{-}RBC\text{-}Th)^N$ anchored by a solitary bacterium at its center. It was found in the same duck. Toroid formation can cause a sufficient amount of intercellular force to result in distortions of the RBC cm as is seen here.

A scatter plot of H/L_2 versus H/L_1 for embryos, ducklings, and older ducks is in Figure 8. Cut-off values are those typically used to separate stress/non-stress hemograms for older avian samples. Neither H/L ratios nor the SDC appears remarkable (Table 1). However, as some of the data are from embryo and hatchling blood samples, it is recognized that the 0.4/0.5 H/L cut-off values are tentative.

Table 1. Standard differential counts in percentage based on four blood films each from embryonic day 24 and day 1 commercial duckling stock (2 × 200 cells/count).

Percentage	HT	HV	HC	Ls	Lm	NK	Bst	Mn	Ba	Eo	RT	Total	TWBC (K)	H/L 1	H/L 2	Δ H/L
Ed24	16.3	0.0	13.5	44.4	10.0	0.0	2.0	0.2	12.8	0.7	0.4	100	7.3	0.7	0.6	0.1
d1	33.3	0.0	32.1	24.3	5.3	0.0	0.4	0.0	3.9	0.7	0.4	100	45.6	2.8	2.4	0.5

H: Heterophil (HT typical, HV variant, HC classic) Ls: Small lymphocyte ~6µm diameter, Lm: Medium and large lymphocyte (diameter 8 - 10 µm), NK: Natural killer, Mn: Monocyte, Ba: Basophil, bst: Blast cell, RT: Reactive thrombocyte, Eo: Eosinophil. H/L 1 = (HT+HV+HC)/Ls; H/L 2 = (HT+HV+HC)/(Ls + Lm), ΔH/L = H/L 1- H/L 2; TWBC total white blood cells per cubic µL in thousands (K)

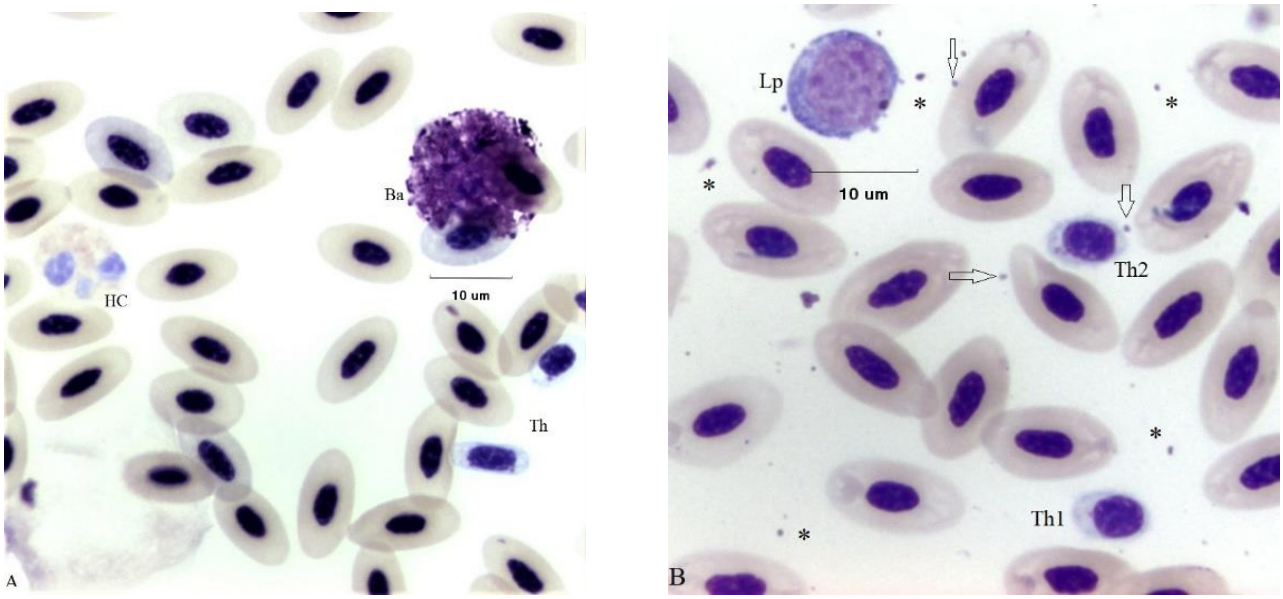

Figure 1. Normal thrombocytes (**A**). A pair of normal (quiescent) Th in a field with mature RBC, duckling at 1d. Each has 1 or 2 magenta specific granules, with A_C ~ 20 µm^2. An atypical heterophil (HC) and a reactive basophil (Ba) are nearby. Thrombocyte in a field also with bacteria (**B**).

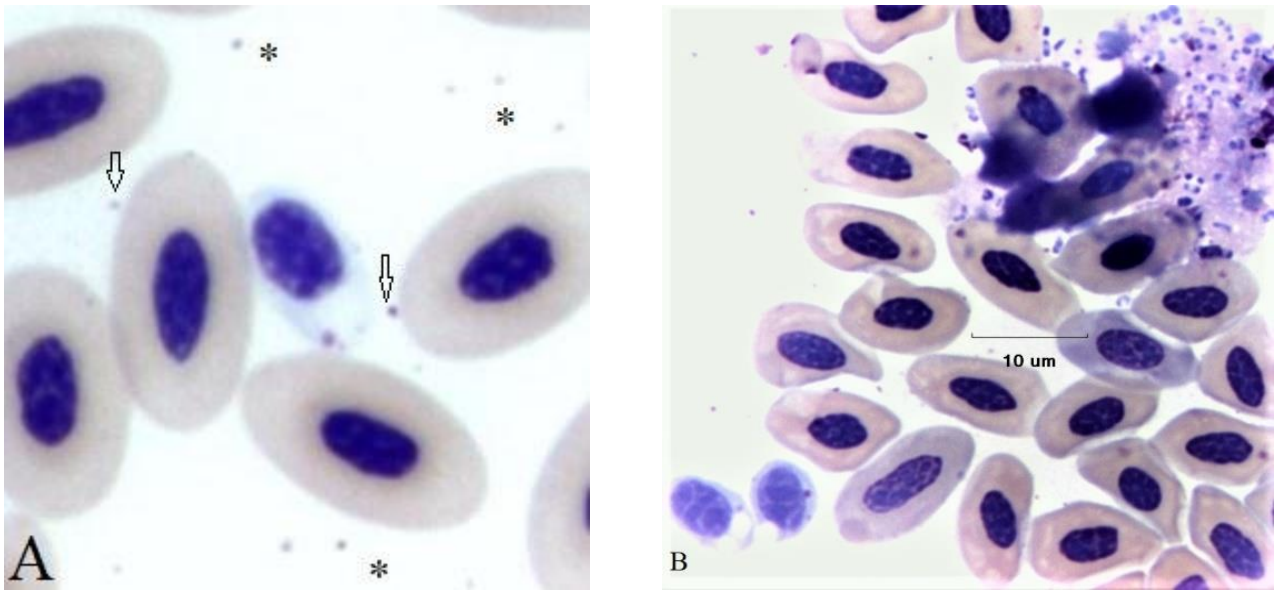

Figure 2. Reactive thrombocytes (**A**). Reactive thrombocyte with a conspicuous cytoplasmic vacuole, in a field with CAB (arrows) and free encapsulated bacteria (*). Reactive thrombocytes with cytoplasmic vacuoles and Th-Th attachment in a field with a polymicrobial colony (**B**); duckling d1.

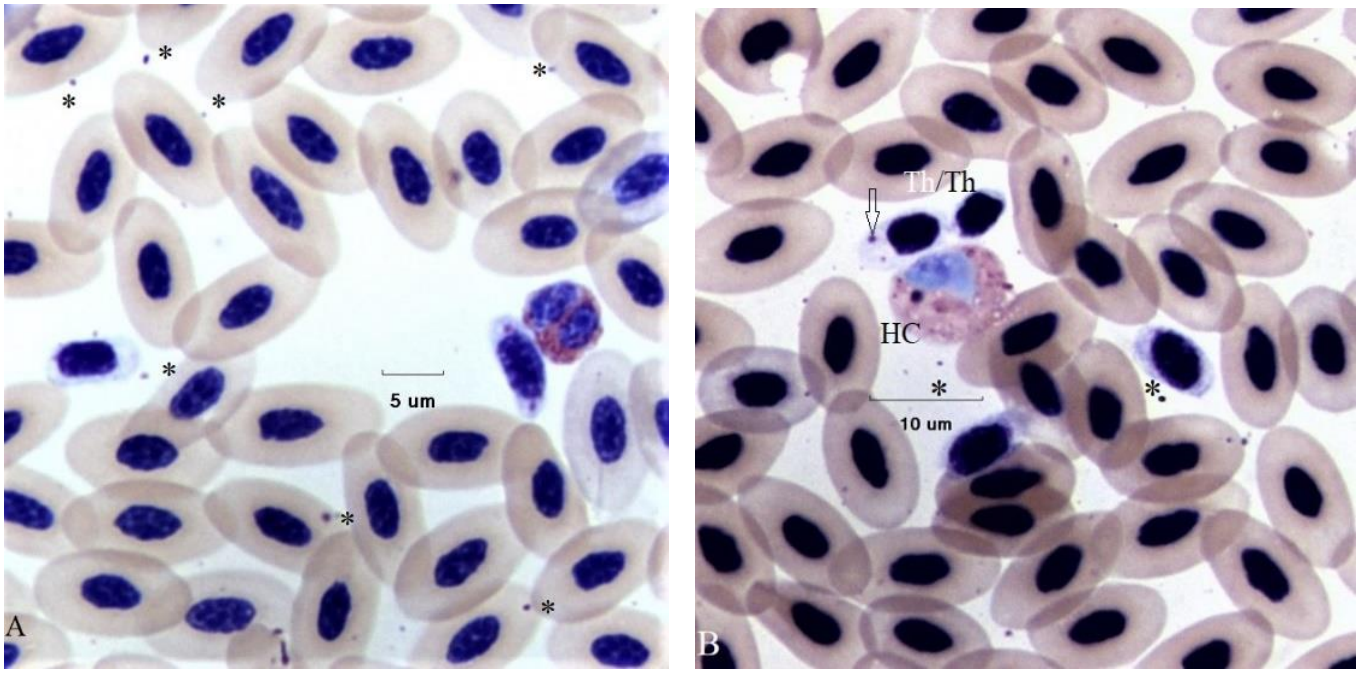

Figure 3. Thrombocyte attachments (**A**). The adhesive phase of thrombocyte reactivity can result in attachment to another thrombocyte or a cell of another series; a small eosinophil (Th/Eo). CAB (*) are also present (Ed24). An example of phagocytic (arrow) Th/Th attachment in a field with thrombocyte overlapping RBC and free bacteria (*). HC is an atypical classic (phagocytic) heterophil (**B**).

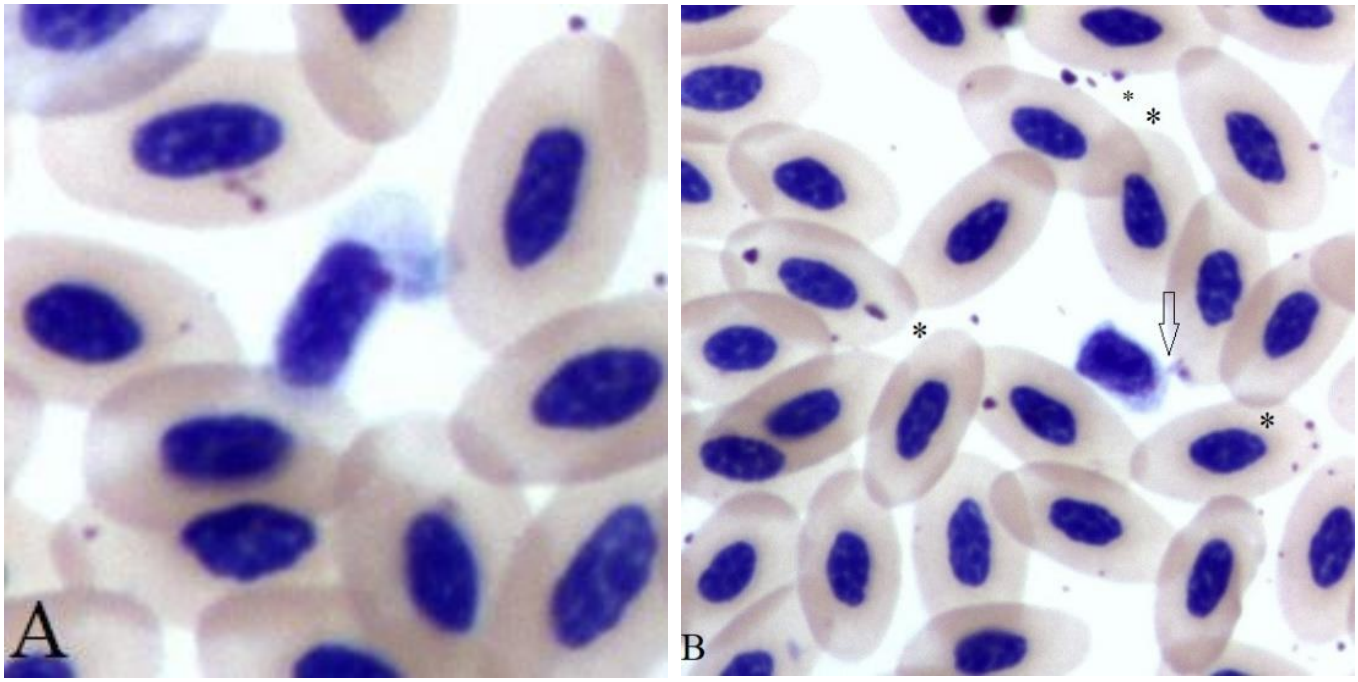

Figure 4. Cytoplasmic loss (**A**). Examples of reactive thrombocyte with partial (panel A) and more advanced loss of cytoplasm (panel **B**) found in the same sample (d1 duckling). CAB (arrow) and free bacteria (*) are also evident.

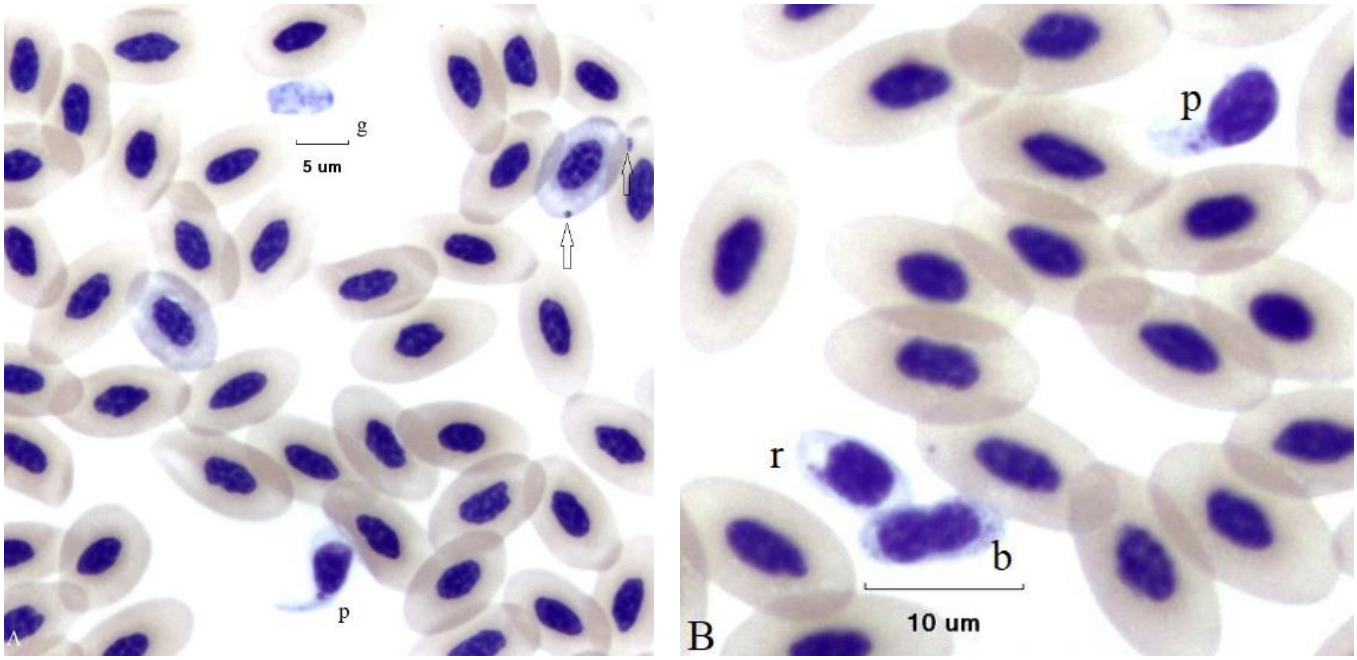

Figure 5. Pseudopod types (**A**). An example of a pseudopod type reactive thrombocyte in CAB fields. Multiple reactive thrombocyte in a single field: pseudopod (p) vacuolate (v) and (b) binuclear (Ed24, **B**).

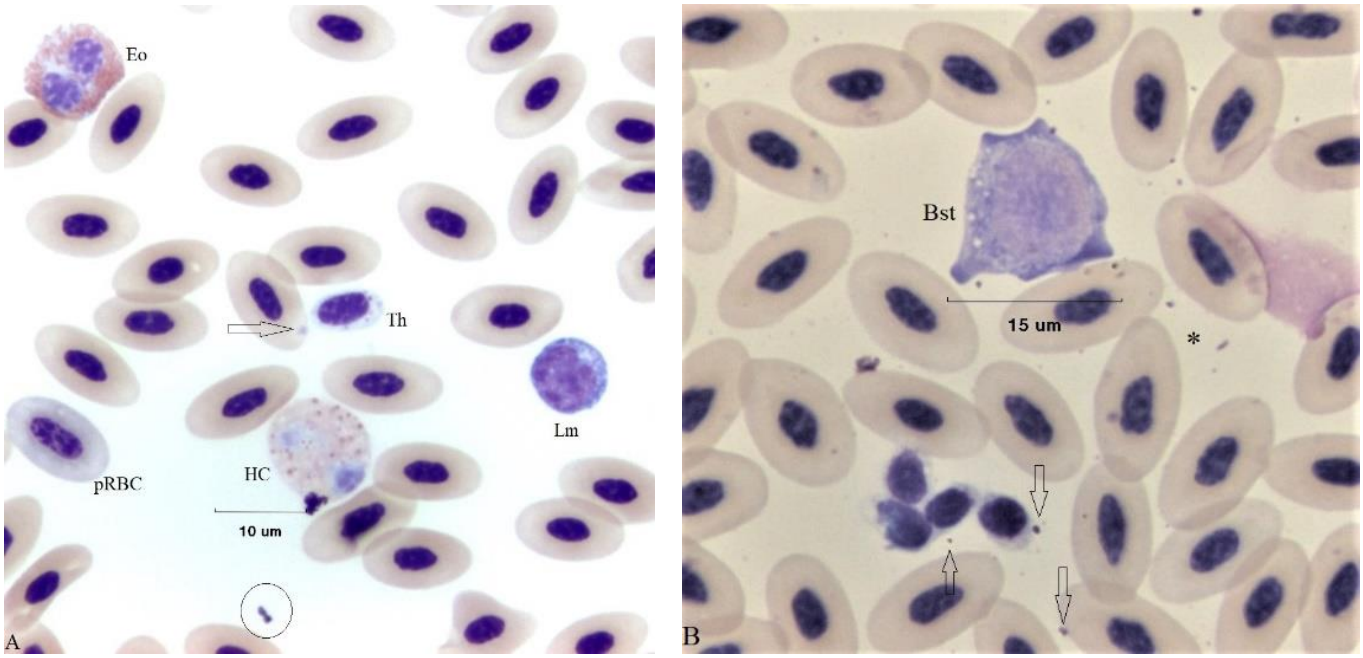

Figure 6. Thrombocytes accompanied by atypia of another series (**A**). Reactive thrombocyte in a field in a hatchling (d1) with an atypical heterophil (HC) an eosinophil (Eo) a reactive plasmacytoid lymphocyte (Lm) CAB (arrow) free bacteria (circle). Aggregated thrombocyte in a (Ed24) field with free (*) and CAB (arrows) and a polyploid blast cell (Bst, **B**).

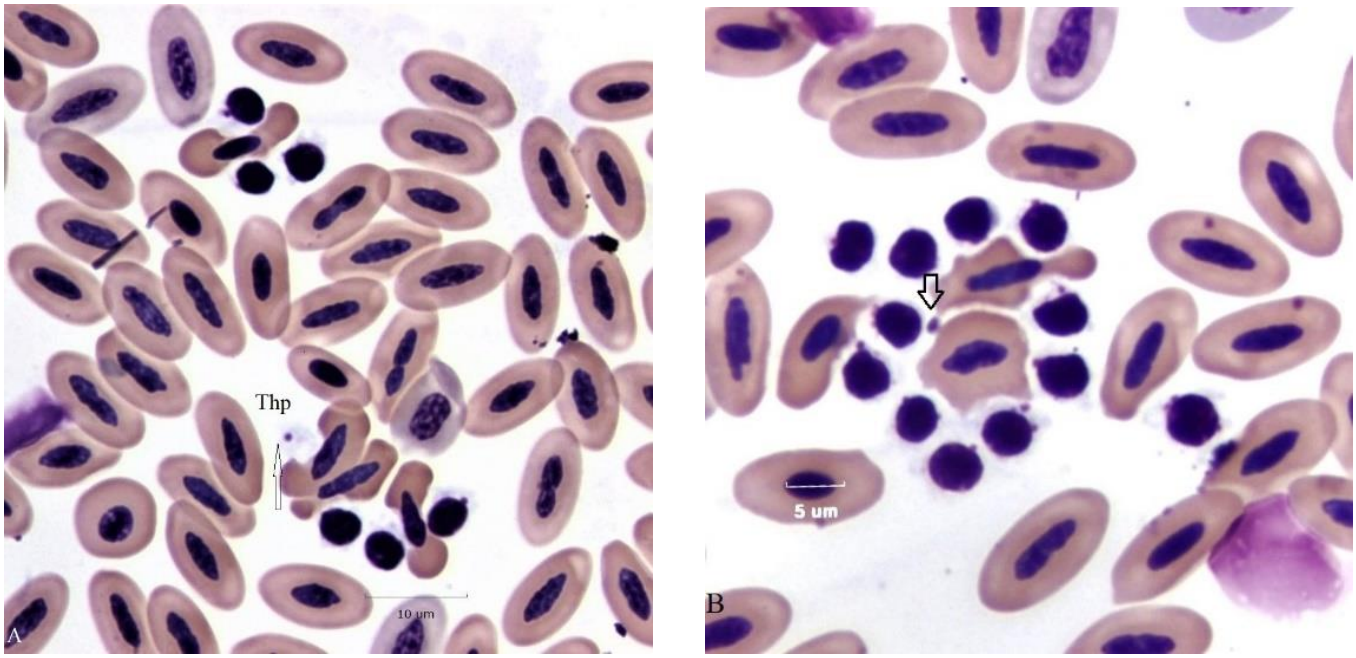

Figure 7. Toroid formation (**A**). Th-RBC toroid a phagocytic thromboplastid (Thp) containing an encapsulated bacterium (arrow). A top, a simple toroid, a more complex type. A multilayered toroid (Th-RBC-Th)^{N} in the same breeding duck at 59 weeks. A bacterium is located at the toroid center (arrow, **B**).

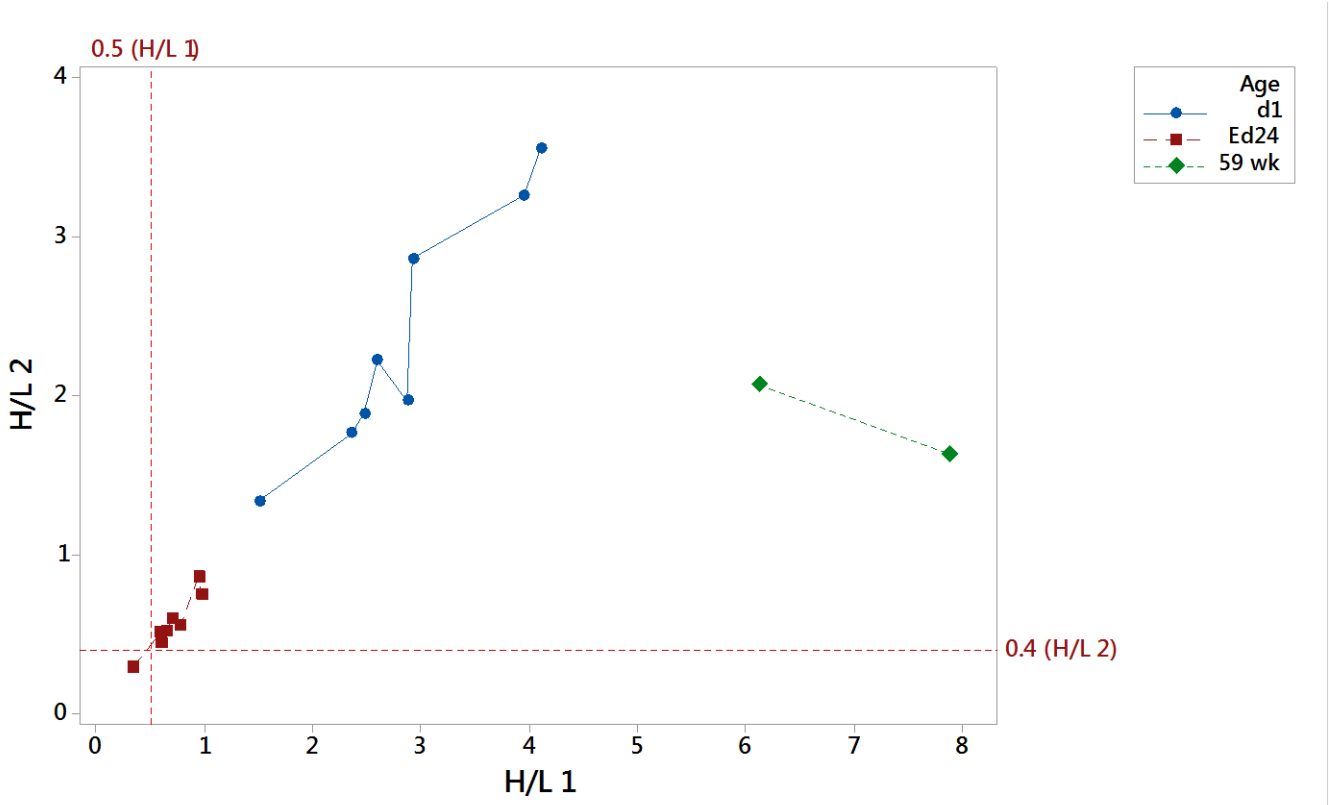

Figure 8. Scatter plot with stress/non-stress cut-offs of duplicate H/L 2 (0.4) vs. H/L 1 (0.5) of duckling blood computed from 2 x 200 cell SDCs sampled at Ed24 and d1. Wk 59 values are means (N=5).

DISCUSSION

The present observations indicated that the transition of quiescent thrombocytes to the reactive state was accompanied by a series of morphological changes some of which were recognizable at the level of ordinary light microscopy. Factors causing these changes are likely related to the presence of bacteria and their molecular products. These are assumed to influence the hemogram independently from factors influencing the H/L ratio. These included the development of vacuoles, an increase of specific granules, and the development of a capacity for adhesion to other thrombocytes and cells of another series. Some of these changes paralleled those described by Gross (1989) who studied crystal violet-stained thrombocytes at a lower magnification with an hemacytometer. Based on the present observations and additional studies (Cotter, unpublished) vacuoles as detected by light microscopy are distinct from the Golgi seen with the electron microscope. Chicken thrombocytes with both a Golgi and vacuoles, located at opposite poles, have been described using electron microscopic (EM) techniques (Daimon and Uchida, 1982). Presumably, vacuoles occur due to the accumulation of secretory products earlier assembled at the Golgi.

Reactive thrombocytes are often found in the same fields as CAB and free bacteria. Given the recognition of the phagocytic ability of thrombocytes and the importance of phagocytosis in bacterial defense, these observations are consistent with earlier *in vitro* observations (Carlson et al., 1968; Wigley et al., 1999). A monoclonal antibody (K1) described by Kaspers et al. (1993) reacted with the surface of both macrophages and thrombocytes. The K1-reactive surface receptor substance may function as an attachment site in the phagocytic response; a property shared by both cell types. The metastasis of mammalian tumors by extravasation is facilitated by platelets (Schlesinger, 2018). The elaboration of pseudopods (Figure 5A, B) is an early manifestation of the means where other (inflammatory) cells cloaked with thrombocytes begin the extravasation process.

Toroids as described here appear to challenge a suggestion that avian thrombocytes are less likely than mammalian platelets to form vaso occlusive emboli due to lower levels of $\alpha(_2b)\beta_3$ integrin (Schmaier et al., 2011). Toroids can become even larger than those in Figure 7A, B and therefore occlude limb vessels; predisposing to the development of lameness and other leg problems. Triggering receptors expressed on myeloid cells (TREM) family receptors are known to be expressed on the thrombocytes surface. These Ig-like molecules imbedded in the cm are thought to have soluble versions as well (Turowski et al., 2016). Toroids may result from binding of bacteria between the anchored Ig molecule with the soluble form adsorbed to the RBC surface (Figure 7, B).

Most reactive thrombocytes were accompanied by free or CAB. As duck embryos are about to begin the shell-pipping stage of incubation between Ed24 and Ed25, bacteria located on the shell could easily find an opportunity to gain entrance during this period. This would also account for bacteria and some of the reactive thrombocytes in hatchling samples. Moreover, there is an opportunity for bacteria to enter the egg shell and the egg proper through its pores at any time after the egg is laid. Moreso, if environmental conditions allow and good egg handling procedures are not in practice. Shell moisture can offer a vehicle for bacteria to move into pores.

Differentiation of a reactive thrombocyte from a normal physiologic variant may be problematic. As variation in thrombocyte morphology described here was invariably accompanied by reactive cells and atypia of another series (Cotter, 2021) the transition to a reactive state is likely a dynamic response only partially amenable to a microscopic study.

The occurrence of reactive thrombocytes in ducklings whose hemograms and H/L ratios are otherwise normal or unremarkable (Figure 8) draws additional attention to the weakness of relying strictly on a derivative statistic, an undefined H/L, to determine stress status (Lentfer et al., 2015).

CONCLUSION

The present observations suggest the analysis of the avian hemogram may benefit from including the condition of the thrombocyte as well as (atypical) cells of other series. This suggestion is justified because thrombocyte have roles in immunity beyond hemostasis. It is a cell that is often overlooked in determining stress levels or in evaluating the immune status of birds subjected to various experimental treatments. The accompaniment of activated/reactive thrombocyte by CAB and free bacteria should not be overlooked.

DECLARATION

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Competing interests: None.

Ethical considerations

Plagiarism, permission to publish, misconduct, data falsification and double publication or submission, and redundancy have been checked by the author.

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