ULTRASTRUCTURE OF ERYTHROCYTIC STAGES OF AVIAN *PLASMODIUM* SPP. OF THE SUB-GENUS *NOVYELLA* AND ITS "GLOBULE"

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Summary:

A globule frequently refractory, appearing blue, pale blue, or white with Giemsa stain, is characteristic of the intraerythrocytic stages of the type species and of most of the other species included at present in the sub-genus *Novyella*. This globule is absent from the other *Plasmodium* sub-genera. An ultrastructural study has been performed on schizogonic stages of *Plasmodium merulae* from the blood of the blackbird, *Turdus merula*. In section the globule contains a finely granular substance suggesting a condensed or coagulated substance. It differs distinctly from a classical food vacuole by denser contents, and show in some sections (19, 23) a peripheral opaque rim with a radial arrangement of ribosomes. Except for the presence of the globule, in other details *P. merulae* do not diverge from the ultrastructure common to the intra-erythrocytic stages of avian *Plasmodium*.

KEY WORDS: blackbird, *Plasmodium merulae*, sub-genus *Novyella*, globule, ultrastructure.

Résumé : ULTRASTRUCTURE DES STADES ÉRYTHROCYTAIRES DE *PLASMODIUM* AVIAIRES DU SOUS-GENRE *NOVYELLA* ET DE LEUR "GLOBULE"

Les stades intra-erythrocytaires des Plasmodium *du sous genre* Novyella *contiennent dans leur cytoplasme, un globule, le plus souvent réfringent, se colorant avec le Giemsa en bleu, bleu pâle ou blanc. Ce globule n'existe pas chez les autres sous-genres de* Plasmodium *aviaires. Une étude ultrastructurale sur des stades sanguins de* Plasmodium merulae *chez le merle noir,* Turdus merula *a été effectuée. Le globule contient une substance granulaire, homogène, assez dense, évoquant une substance coagulée ou condensée. Il diffère des vacuoles alimentaires classiques par sa plus forte densité, son rebord plus opaque sur certains clichés (19, 23) et la disposition radiaire des ribosomes périphériques. Le globule mis à part, l'ultrastructure de* P. merulae *ne diffère pas de celle connue chez les autres* Plasmodium *d'Oiseaux.*

MOTS CLÉS : *merle,* Plasmodium merulae, *sous-genre* Novyella, *globule, ultrastructure.*

INTRODUCTION

Parasites in the sub-genus *Novyella* were defined by Corradetti *et al.* (1963) as having small schizonts, very little cytoplasm, elongated gametocytes and exo-erythrocytic schizogony in the reticulo-endothelial system but not in erythroblasts. The type species is *Plasmodium vaughani* Novy and MacNeal, 1904, described from *Turdus migratorius* Linnaeus, 1766 in Michigan. A globule, frequently refractory, appearing white, pale blue or blue in Giemsa-stained preparations, can be seen in the intra-erythrocytic stages of the developing parasites of the type species and in most of the other species included in the sub-genus *Novyella*. It is absent from all the other sub-genera. The presence of the globule has been reported in many descriptions of *Plasmodium* (*Novyella*) spp. (Manwell, 1935; Mohammed, 1958; Corradetti & Scanga, 1972; Valkiūnas 2005; Paperna *et al.*, 2008; Paperna *et al.*, submitted). It remains unchanged throughout the development of the parasite and as a residue when the schizont matures. In light microscopy the globule appears totally different from the transparent vacuoles that are frequently seen in *Plasmodium*. Its nature and role are unknown. In this communication we report results of an ultrastructural study performed on blood stages of *Plasmodium merulae* Corradetti and Scanga, 1972 from blackbird.

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MATERIALS AND METHODS

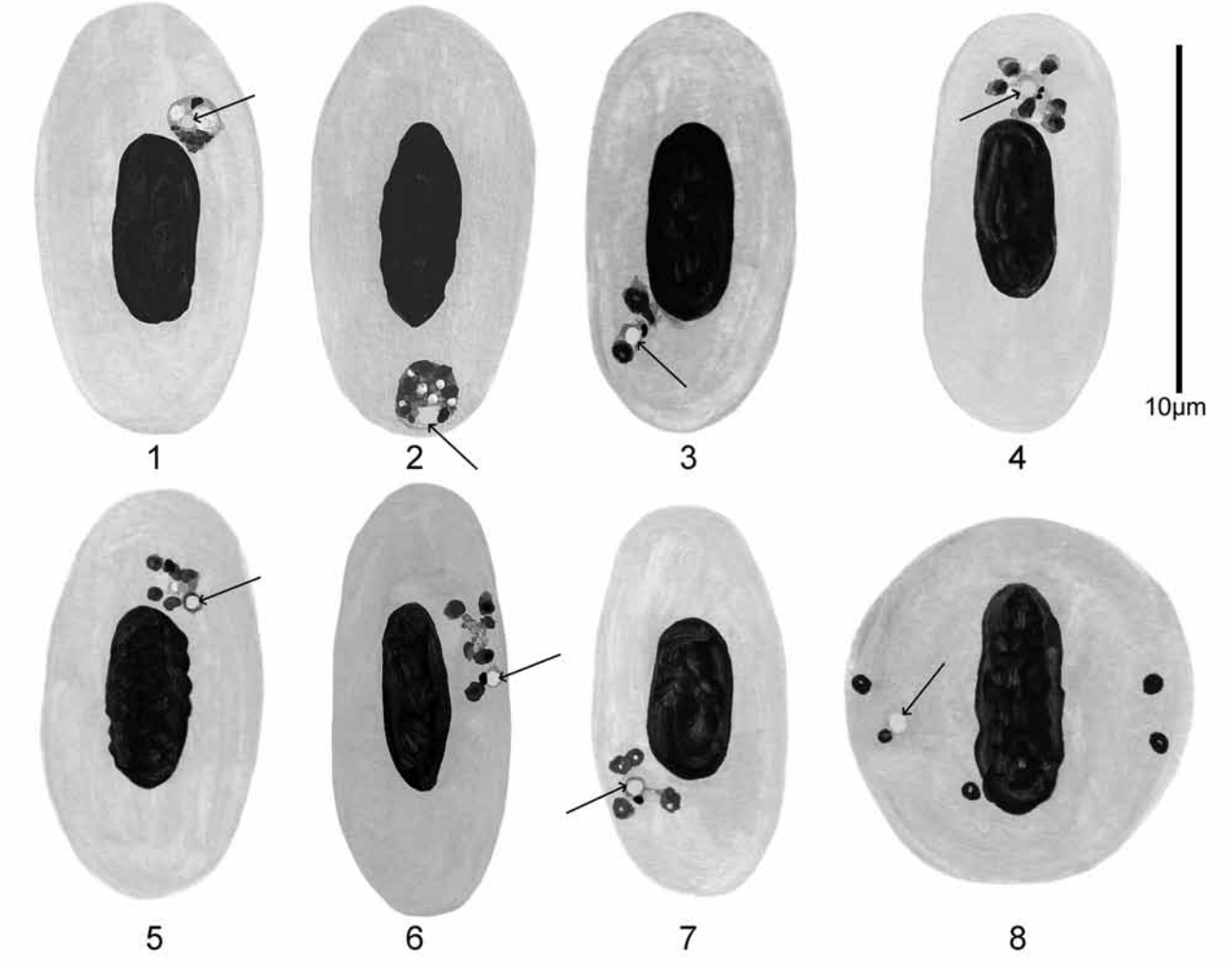
Blackbirds, *Turdus merula* Linnaeus, 1758, were caught by mist net in Luynes (N47°23'07", 0°33'42.5"; Indre-et-Loire; France). The trapped bird was ringed on the right tarsus with a metal numbered ring (of the MNHN of Paris) and morphometric data was recorded. The brachial vein was pierced by a

sterile fine needle and a blood sample was withdrawn into a hematocrit capillary, then the bird was released. To verify infection, drops of blood were smeared onto clean glass slides. Air-dried smears were flooded with absolute methanol and stained for 1 h with 10 % Giemsa (Merck product), in pH 7.4 buffer phosphate.

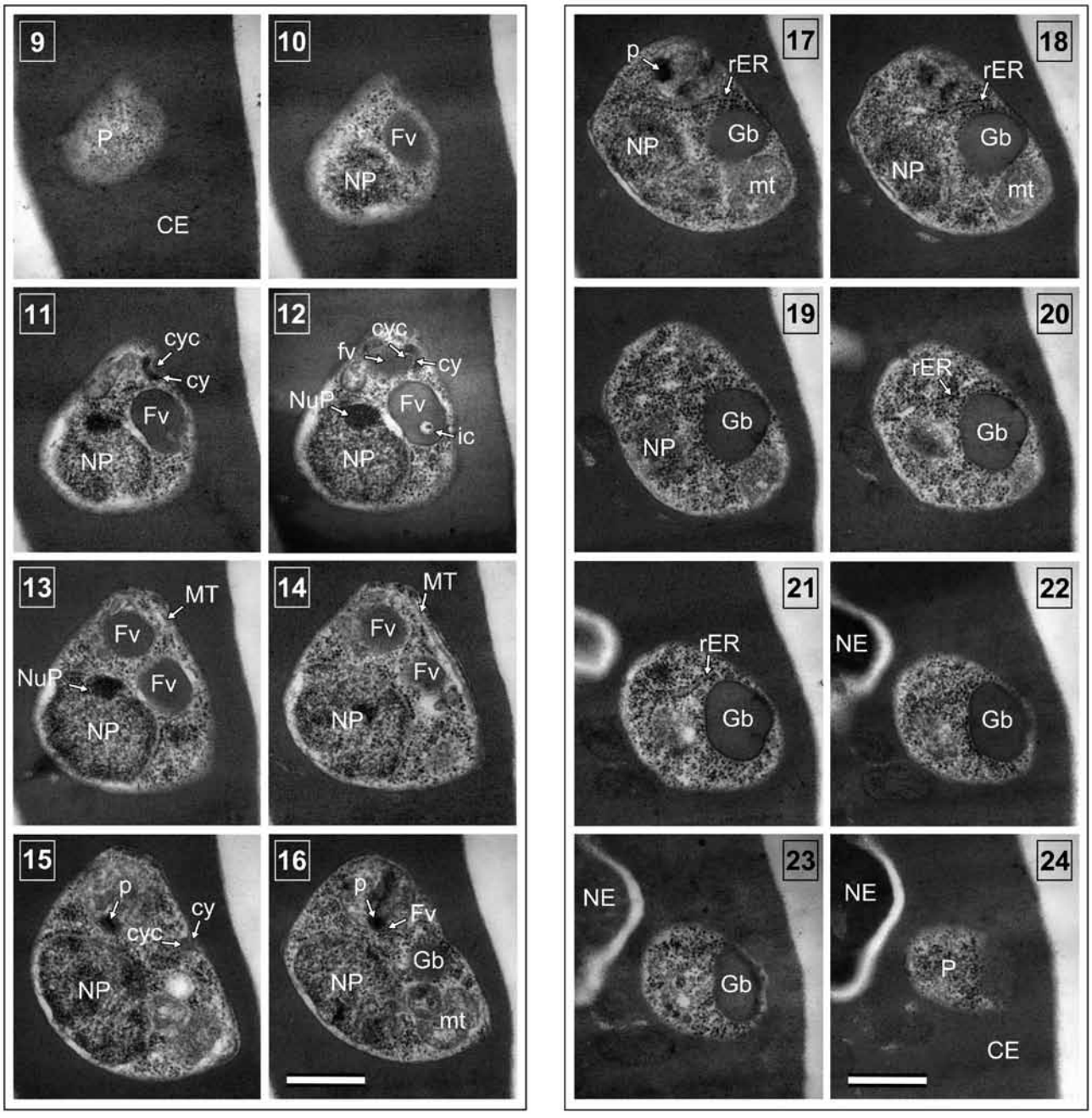
For the electron microscope study a blood sample from one of the positive birds was fixed by incubation for 48 h in 4 % paraformaldehyde and 1 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and post-fixed by incubation for 1 h with 1 % osmium tetroxide. Samples were dehydrated in graded ethanol and propylene oxide series and embedded in Epon resin which was allowed to polymerize for 48 h at 60° C. Serial ultrathin (75 nm) sections were cut on a Leica ultramicrotome, placed on EM grids coated with Formwar membranes, stained with 4 % uranyl acetate (20 min) and 1 % lead citrate (5 min), then observed with a JEM 1011 electron microscope (Jeol, Tokyo, Japan) equipped with Gatan digital camera driven by Digital Micrograph software (Gatan, Pleasanton, CA USA). The scanned pictures were aligned using Adobe Photoshop software prior to being processed for the 3-D reconstruction. The program used, IMOD, was developed by Kremer *et al.* (1996), Bolder University, Colorado.

RESULTS

Viewed in the light microscope, the mature erythrocytic schizont of *Plasmodium merulae* is composed of four to six nuclei held in a reticulate or sparse frame of cytoplasm which also contains large refractile globule and pigment granules



Figs 1-8. – Drawings of Giemsa-stained *Plasmodium* of the sub-genus *Novyella* from different localities in France (Arrow = blue globule). Fig. 1: young trophozoite with globule and two transparent vacuoles from Luynes (Indre-et-Loire). Fig. 2: immature schizont with globule and several small transparent vacuoles from Luynes. Fig. 3: binucleated schizont with blue globule from Seninghem (Pas-de-Calais). Fig. 4: maturing schizont with six nuclei, central globule and transparent vacuoles from Mont-Bernanchon (Pas-de-Calais). Fig. 5: maturing schizont with five nuclei, bulging globule and transparent vacuoles from Mont-Bernanchon. Fig. 6: mature schizont with six nuclei and blue globule from Luynes. Fig. 7: mature schizont with four nuclei and central blue globule from Mont-Bernanchon. Fig. 8: schizont ruptured inside the red-blood cell, residual pigment and globule from Gray (Haute-Saône).



Figs 9-24. – TEM electron micrographs of serial section of a trophozoite of *Plasmodium merulae* from Luynes. Scale bar = 0.2 µm. Fig. 9: section through the parasite cytoplasm. Fig. 10: section showing nucleus of parasite (NP) and food vacuole (Fv). Fig. 11: section showing cytostome (cy), cytostomal cavity (cyc), next to a nucleus (NP) and one food vacuole (Fv). Fig. 12: one food vacuole (Fv) with an inclusion (ic), adjacent to the nucleus of parasite (NP) with nucleolus (NuP); the cytostome (cy) connected to a cytostomal cavity (cyc) and a food vacuole (fv). Fig. 13: section showing nucleus of parasite (NP) with nucleolus (NuP) and two food vacuoles (Fv). Fig. 14: section showing microtubule (MT), nucleus of parasite (NP), two food vacuoles (Fv). Fig. 15: section with cytostome (cy), cytostomal cavity (cyc), food vacuole with pigment contents (p) and nucleus of parasite (NP). Fig. 16: section showing the globule (Gb), a food vacuole (Fv) with pigment contents (p), nucleus of parasite (NP) and lobulated mitochondrion (mt). Fig. 17: section showing a globule (Gb), pigment (p), nucleus of parasite (NP), rough endoplasmic reticulum (rER) and mitochondrium (mt). Fig. 18: section with a globule (Gb), nucleus of parasite (NP), mitochondrium (mt) and rough endoplasmic reticulum (rER). Fig. 19: section with only globule (Gb) and nucleus of parasite (NP). Figs 20, 21: section with globule (Gb) and rough endoplasmic reticulum (rER). Figs 22, 23: section showing globule (Gb) and the nucleus of the erythrocyte (NE). Fig. 24: section showing the parasite (P) inside the cytoplasm of the erythrocyte (CE) and the the erythrocyte nucleus (NE).

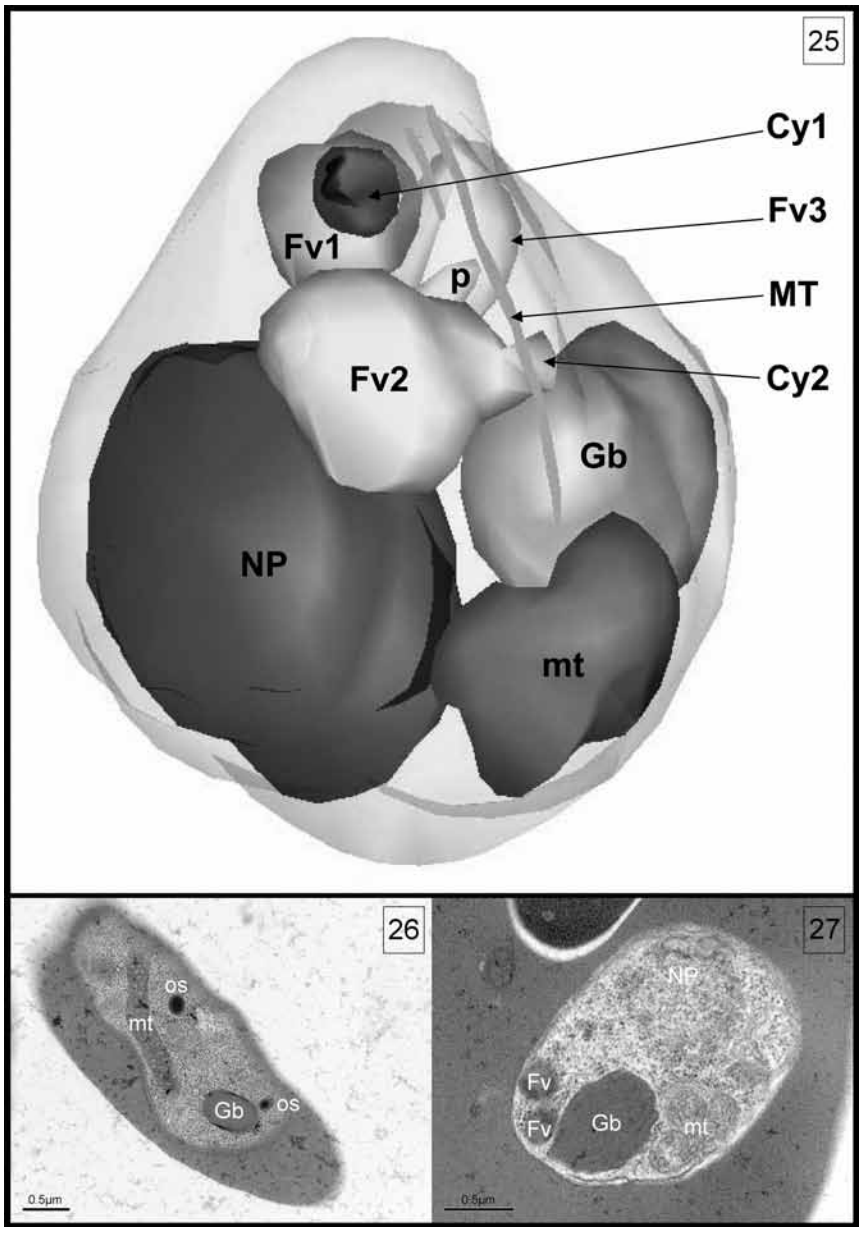


Fig. 25. – Three-dimensional reconstruction of the serial section (Figs 9-24) of a trophozoite of *Plasmodium merulae* from Luynes. Different organelles are visible: the globule (Gb), food vacuoles (Fv1, Fv2) cytostomes (cy1, cy2) and Fv3 with pigment (p); nucleus of the parasite (NP), microtubules (MT) and mitochondrium (mt).

Figs 26, 27. – TEM micrographs of sections of two other trophozoites from the same material. Fig. 26: section with bounded globule (Gb), osmiophilic bodies (Os) and elongate mitochondrion (mt). Fig. 27: section with two food vacuoles (Fv), large globule (Gb), membranous binding not conspicuous, outlines of a nucleus (NP), mitochondrion (mt).

(Figs 1-8). The globule is visible from the youngest stage of the parasite: trophozoite (Fig. 1) and very young schizont (Fig. 2). It stays during all the maturation of the schizont (Figs 3-7) and persists in the ruptured schizont (Fig. 8).

All electron micrographs were of uninucleated parasites (young trophozoites as evidenced by the presence of residual microtubules). They show a single nucleus with a surrounding narrow cytoplasmic layer (Fig. 12), bound by a membrane (Fig. 14) and beneath, the microtubules (Fig. 14). Other ultrathin images reveal sections of cytoplasm only (Figs 9, 24). The globule appears as a round body with homogenous (fine granular) contents of medium electron density with electron dense rims (Figs 16-23, 26, 27). The globules are often seen adjacent to a nucleus (Figs 16-19, 27). Some sections reveal one (Figs 10, 11) or two food vacuoles (Figs 13, 14, 27). One section (Fig. 12) show one food vacuole containing a translucent inclusion and one forming food vacuole. Some sections reveal cytostome, cytostomal cavity (Figs 11, 12, 15) and food vacuole with presumably pigment material (Figs 15, 16). The cytoplasm is densely packed with ribosomes and rough endoplasmic reticulum (Figs 17, 18, 20, 21) and sometimes reveals subpellicular microtubules (Figs 13, 14). Much of the cytoplasmic space is occupied by round or elongate, single or splitted mitochondria (Figs 16, 17, 18, 26, 27), one section shows osmiophilic bodies (Fig. 26).

DISCUSSION

Ultrastructural accounts of erythrocytic stages are available for a number of avian species of *Plasmodium*: *P. cathemerium, P. fallax, P. lophurae* (Aikawa, 1966) and *P. elongatum*, (Aikawa *et al.*, 1967; Aikawa & Sterling, 1974); but none from species which have been included in the sub-genus *Novyella*. In none of the available fine structural images of erythrocytic schizonts from the above mentioned species could be traced a structure comparable to that which has been identified as a globule.

With the light microscope the globule is distinctly different from a classical vacuole. After fixation by the methanol and staining by Giemsa a vacuole appears as a transparent round structure while the globule as a refractile sphere, often bluish. Its size changes little during the parasites maturation and it remains intact even when the schizont ruptures. It is a characteristic structure of a group of small bird *Plasmodium* (*Novyella*) (Landau *et al.*, 2010) and its type species, *Plasmodium vaughani*.

Ultrastructurally, the globule is reminiscent of a food vacuole. However, it differs by its higher density, particularly at the periphery, and the radial arrangement of ribosomes.

Except for the presence of the globule, in other details the main ultrastructural features of the trophozoites show few differences from those of the intra-erythrocytic stages of other species of avian *Plasmodium*. Martinsen *et al.* (2006) sequenced a group of species from birds in the USA, recognized to belong to the sub-genus *Novyella* by their small schizonts and their scanty cytoplasm. They clustered separately from species belonging to the other sub-genera: *Haemamoeba*, *Huffia* and *Bennettinia*. Two isolates were even completely separated from the rest. Later morphological examinations of specimens from the species identified as *Novyella* have shown that all had one or several globules (Paperna & Martinsen, unpublished).

In conclusion, the presence of this globule is in our opinion a definitive marker for avian *Plasmodium* of the sub-genus *Novyella*.

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REFERENCES

AIKAWA M. The fine structure of the erythrocytic stages of three avian malarial parasites, *Plasmodium fallax, P. lophurae* and *P. cathemerium*. *American Journal of Tropical Medicine and Hygiene*, 1966, *15*, 449-471.

AIKAWA M., HUFF C.G. & SPRINZ H. Fine structure of the asexual stages of *Plasmodium elongatum*. *Journal of Cell Biology,* 1967, *34*, 229-249.

AIKAWA M. & STERLING C.R. Intracellular Parasitic Protozoa. Academic Press Inc. New York, San Francisco, London, 1974, 76 pp.

CORRADETTI A. GARNHAM P.C.C. & LAIRD M. New classification of the avian malaria parasites. *Parassitologia,* 1963, *5*, 1-4.

CORRADETTI A. & SCANGA M. *Plasmodium (Novyella) vaughani* subsp. *merulae*, n. subsp., parasita di *Turdus merula*, con descrizione del ciclo pre-erythrocytico. *Parassitologia*, 1972, *14*, 85-93.

CORRADETTI A. & SCANGA M. The *Plasmodium vaughani* complex. *Experimental Parasitology*, 1973, *34*, 344-349.

LANDAU I., CHAVATTE J.-M., PETERS W. & CHABAUD A. The subgenera of avian *Plasmodium*. *Parasite*, 2010, *17*, 3-7.

MANWELL R.D. *Plasmodium vaughani* (Novy and MacNeal). *American Journal of Hygiene* 1935, *21*, 180-187.

MARTINSEN E.S., WAITE J.L. & SCHALL J.J. Morphologically defined subgenera of *Plasmodium* from avian hosts: test of monophyly by phylogenetic analysis of two mitochondrial genes. *Parasitology*, 2007, *134*, 483-490.

MOHAMMED A.H.H. Systematic and experimental studies on protozoal blood parasites of egyptian birds. Cairo University Press, Cairo, 1958, *1*, 165pp.

NOVY F.G. & MACNEAL W.J. Trypanosome and bird malaria. *American Medicine*, 1904, *8*, 932-934.

PAPERNA I., YOSEF R., CHAVATTE J-M., GILL H. & LANDAU I. Species of *Plasmodium* of passerine birds with four nuclei, with description of new species. *Acta Parasitologica*, 2008, *53*, 227-236.

PAPERNA I., YOSEF R., CHAVATTE J-M. & LANDAU I. Malarial infections in resident and migratory passerine birds in Israel: description of new species (Submitted).

VALKIŪNAS G. Avian malaria parasites and other haemosporidia. CRC Press Boca Raton, London, New York, Washington D.C., 2005, 946 pp.

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