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Hematology, Morphology and Ultrastructure of Blood Cells and Blood Parasites from Puff-faced Watersnakes (*Homalopsis buccata*)

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ABSTRACT

Blood samples of 45 puff-faced watersnakes (*Homalopsis buccata*) in the Queen Saovabha Memorial Institute were collected from ventral caudal vein for both basic hematology and light microscopic, scanning and transmission electron microscopic features of blood cells. Seventeen samples (37.8%) were positive for hematozoa infections. The single infection of *Hepatozoon* sp., trypanosome and *Haemogregarina* sp. was found in 4, 4 and 5 snakes respectively. The other four snakes were infected by both trypanosome and *Hepatozoon* sp. There were no significant differences of all hematological value between the hematozoa-negative and the hematozoa-positive snakes except fibrinogen concentration which was found higher in the negative group. Lymphocytes were the most commonly observed leukocytes and average 6-8 μm in diameter. Azurophils were the second most commonly observed leukocytes, average 10-17 μm in diameter and might play a major role in eliminating the trypanosome. Heterophils were the largest leukocytes, average 16-19 μm in diameter and the third commonly observed leukocytes. Eosinophils usually were medium-sized cells, average 10-14 μm in diameter but in some occasion the very large cells were also detected. Basophils were smaller than heterophils and eosinophils. Scanning electron microscopy revealed the membrane surfaces of normal and abnormal erythrocytes, *Hepatozoon* sp. infected erythrocytes, thrombocytes, eosinophil and trypanosomes. Transmission electron microscopy revealed the organelles within azurophil, eosinophil, heterophil and trypanosome.

Key words: Electronmicroscopy, *Haemogregarina*, Hematology, *Hepatozoon*, *Homalopsis buccata*, puff-faced watersnakes, trypanosome

INTRODUCTION

Puff-faced or mask-faced watersnake (*Homalopsis buccata*) is immediately identified by the large broad head and a white, mask-like pattern on the top of the head. It consumes fish and frogs. Its habitat is commonly found in most of Southeast Asia (Cox *et al*., 1998). The Queen Saovabha Memorial Institute (QSMI) had initiated a captive breeding program since 1994 to supply healthy snakes for venom and antivenom production. These venomous snakes prey on mice and occasionally on puff-faced watersnakes. The venomous snakes were highly infected with *Hepatozoon* sp. (Salakij *et al*., 2001). Reptile blood cell morphologic characteristics are heterogeneous. Variations in cell characteristics and cell populations were existed between species within the order Squamata (Alleman *et al*., 1999).

The purpose of this study was to obtain the hematological data, characterization of blood cells

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and compare if the hematozoas in the puff-faced water snakes were the same as those found in the venomous snakes.

MATERIALS AND METHODS

Blood samples of 45 puff-faced watersnakes were collected from ventral caudal vein during January to March 2000. Blood smears were prepared immediately and air-dried. They were stained with Wright's and Wright-Giemsa stained for white blood cell differentiation and hemoparasite examination. The collected blood samples were kept immediately in EDTA, immediately stored at 4°C and processed within 2 hours.

The packed cell volume (PCV) were determined by microcapillary technique. The total solids were measured using a Atago®SPR-N refractometer (Japan). Fibrinogen was calculated as the difference between the total solids before and after incubation for 3 minutes at 56°C and recentrifugation (Jain, 1986). The total red blood cell count (RBC), were determined manually with the improved Neubauer counting chamber after the blood was diluted 200 times with the Natt and Herrick's solution (Natt and Herrick, 1952). The total number of the RBC was counted in the five red blood cell squares of the center large square of the chamber in duplicate. The duplicates were averaged for agreement within 15% difference and multiplied by 10,000 to calculated the number per microlitre (Campbell, 1986). The leukocyte count (WBC) was determined in the same counting chamber as the RBC count except the leukocytes were counted in duplicate from four large squares of the chamber at 40X magnification. The leukocyte neuclei were stained blue whereas the thrombocyte neuclei were unstained or very pale blue. The duplicates were averaged for agreement within 15% difference and multiplied by 500 to calculate the number per microlitre. The hemoglobin concentration (Hb) was determined by the cyanomethemoglobin method which free RBC neuclei were removed by the centrifugation before reading the absorbance (Campbell, 1986).

Blood smears were fixed in methanol and stained with Wright-Giemsa (WG) stain (Benjamin, 1978) for determination of differential leukocyte count, identification of hematozoa infection and morphological evaluation of all blood cells. Grading of *Hepatozoon* sp. and *Haemogregarina* sp. was quantitated by the number of infected erythrocytes as described elsewhere (Salakij *et al*., 2001). A minimum of 200 leukocytes were counted for differential leukocyte determination. For comparison, blood smear from 5 puff-faced watersnakes were stained with one step Wright's staining method that did not required methanol fixation prior to staining stain (Benjamin, 1978).

Blood samples were also prepared for reticulocyte count by staining with new methylene blue using wet preparation (Benjamin, 1978). The percentage of reticulocytes presented in 1,000 erythrocytes was determined. The reticulocytes that contained distinct aggregated reticulum were described as aggregate reticulocytes whereas punctate reticulocytes contained a few small dots (Jain, 1986).

For each parameter obtained, data from hematozoa-negative and positive were calculated for means, variances and standard error using SPSS® for window ™ (Norusis, 1993). Significant difference between means were determined using an independent sample T-test model.

For scanning electron microscopy (SEM), a drop of blood were fixed using 1.5% glutaraldehyde (GA) in 0.1 M phosphate buffer (PB) at 4°C for 24 hr. Specimens were dehydrated through a graded acetone series. Gold-coated smears were examined under Jeol JSM-35CF scanning electron microscopy.

For transmission electron microscopy (TEM), buffy coats were fixed in 2.5% GA (PB) for 24 hr and postfixed in 1% osmium tetroxide. Specimens were dehydrated through a graded acetone series and embedded in Spurr's epoxy

resin. Ultrathin sections, stained with uranyl acetate and lead citrate, were examined using Jeol 1200Ex TEM.

RESULTS

Seventeen samples (37.8%) were positive for hematozoa infections. The single infection was found in thirteen snakes including: *Hepatozoon* sp., trypanosome and *Haemogregarina* sp. The other four snakes (8.9%) were mixed infection between trypanosome and *Hepatozoon* sp. (Table 1). There were no significant differences of all hematological values between the hematozoa-negative and the positive groups except fibrinogen concentration which was higher in the negative group (Table 2).

Erythrocytes were homogeneous in color but moderately anisocytosis (Figure 1, 2, 3). Cytoplasmic holes were detected in less than 1% erythrocytes (Figure 3c). The other shapes of abnormal erythrocytes were seldom detected (Figure 3b, 3c). *Hepatozoon*– and *Haemogregarina*-infected erythrocytes were larger than those non-infected ones (Figure 2b, 2d, 3e). By SEM, erythrocytes were ellipsoidal lacking the doming appearance in the site of the nucleus (Figure 3a, 3b, 3c).

Thrombocytes were elongate and approximately half the size of mature erythrocytes. Under the Wright's stain, cytoplasm was slightly basophilic (Figure 1i) compared with the unstained cytoplasm with azurophil granules in Wright-Giemsa stain (Figure 1a). Thrombocytes were easily differentiated from lymphocytes by the characteristic perinuclear and cytoplasmic vacuolation (Figure 1a, 1i). By SEM, their membranes were more irregular than those of erythrocytes (Figure 3a).

Leukocytes of puff-faced water snakes were categorized into 6 groups; azurophil, heterophil, eosinophil, basophil, lymphocyte and monocyte. For comparison, the blood smears stained with one step Wright's stain provided staining quality for identification of all blood cell type but in Wright's stain the erythrocytes stained more basophilic (Figure 1h, 1i).

Lymphocytes in puff-faced watersnakes were the most prevalent circulating cells (Table 2). They were small, well differentiated and averaged 6-8 μm in diameter (Figure 1a).

Azurophils were the second most commonly observed leukocytes, which contained fine indistinct azurophilic granules. They were round and 10-17 μm in diameter. The nuclei were round to irregular with clump chromatin and located centrally to eccentric (Figure 1b, 1d). Ultrastucturally, they contained numerous membrane-bound granules, some mitochondria and rough endoplasmic reticulum (Figure 4b). Phagocytosis of trypanosome by azurophils was detected only by TEM (Figure 4b). The number of monocytes is very rare and their characters were similar to mammalian monocytes (Figure 1d).

Heterophils were the largest of the leukocytes and average 16-19 μm in diameter. They contained large numbers of irregular shape, dull eosinophilic granules (Figure 1c). By Wright's stain, heterophil granules were easily seen by reddish-orange bright granules (Figure 1h). Ultrastructurally, heterophils contained pleomorphic population of large granules with variable electron density (Figure 4d).

Eosinophils contained numerous round and light blue granules that often occluded visualization of the nucleus (Figure 1e, 1h). They usually were medium-sized cells (10-14 μm in diameter) but in some cases very large cells were also detected (Figure 1f, 1i). By Wright's stain, eosinophil granules were stained dark blue when compared to the heterophils (Figure 1h). By SEM, their granule contour was bulging showing the custard apple-liked appearance (Figure 3d). Ultrastructurally, eosinophils contained homogeneous electron density granules with some cytoplasmic projections (Figure 4c). The eosinophil number were very high both in the negative and positive groups (Table 2).

Basophils were very low, average 9-12 μm in diameter and were slightly smaller than

Table 1 Number and percentage of hematozoa-negative and positive puff-faced watersnakes which was subgrouped according to sex.

	Male	Female	Total	%
Negative	15	13	28	62.2
Hepatozoon sp.	2	2	4	8.9
Trypanosoma sp.	4	0	4	8.9
Trypanosoma sp. and *Hepatozoon* sp.	3	1	4	8.9
Haemogregarina sp.	5	0	5	11.1
Total	29	16	45	100
%	64.4	35.6	100	

Table 2 Comparative hematology (mean ± SE) between hematozoa-negative and positive puff-faced watersnakes.

	Hematozoa-negative	Hematozoa-positive
Number	28	17
PCV (%)	19.6 ± 1.2	22.3 ± 1.6
Hb (g/dl)	6.34 ± 0.38	6.99 ± 0.46
RBC ($x10^6/\mu l$)	0.530 ± 0.046	0.618 ± 0.068
WBC ($x10^3/\mu l$)	12.11 ± 1.00	11.95 ± 1.78
Azurophils ($x10^3/\mu l$)	4.22 ± 0.48	3.96 ± 0.82
Heterophils ($x10^3/\mu l$)	1.91 ± 0.26	1.31 ± 0.17
Basophils ($x10^3/\mu l$)	0.005 ± 0.003	0.02 ± 0.01
Eosinophils ($x10^3/\mu l$)	0.69 ± 0.10	0.70 ± 0.14
Lymphocytes ($x10^3/\mu l$)	5.16 ± 0.70	5.93 ± 1.26
Monocytes ($x10^3/\mu l$)	0.08 ± 0.03	0.03 ± 0.02
Azurophils (%)	35.8 ± 3.0	32.5 ± 3.9
Heterophils (%)	15.3 ± 1.6	13.1 ± 2.1
Basophils (%)	0.07 ± 0.05	0.2 ± 0.09
Eosinophils (%)	6.5 ± 0.9	6.2 ± 0.9
Lymphocytes (%)	41.3 ± 2.8	47.5 ± 4.3
Monocytes (%)	0.8 ± 0.4	0.4 ± 0.2
PP (g/dl)	5.7 ± 0.30	5.6 ± 0.40
Fibrinogen (mg/dl)	142.8 ± 20.8*	64.7 ± 17.0*
Agg. Reticulocytes (%)	0.74 ± 0.30	3.27 ± 1.68
Punct. Reticulocytes (%)	4.11 ± 1.32	9.61 ± 2.83

* Significant difference at $p<0.05$.

eosinophils (Figure 1g). They contained small dark purple staining metachromatic granules that obscure the lobed nucleus.

Trypomastigote form of trypanosomes were large, broad body width (Figure 2a). By SEM, they were often in a cluster (Figure 3f). Ultrastructurally, they contained nucleus, very large mitochondria, abundant ribosomes (Figure 4b), some dense and multivesicular bodies (Figure 4a). The gamonts of *Haemogregarina* sp. were easily defined from those of the *Hepaozoon* sp. by their very large-size and more granularity (Figure 2b). There were two kinds of *Hepaozoon* gamonts; the small (Figure 2c) and the large gamonts (Figure 2d). Both *Hepaozoon* and *Haemogregarina* gamonts were resided in the cytoplasm of enlarged erythrocytes (Figure 3e) and displaced the nucleus (Figure 2b, 2c). Some gamonts were free from erythrocytes within their

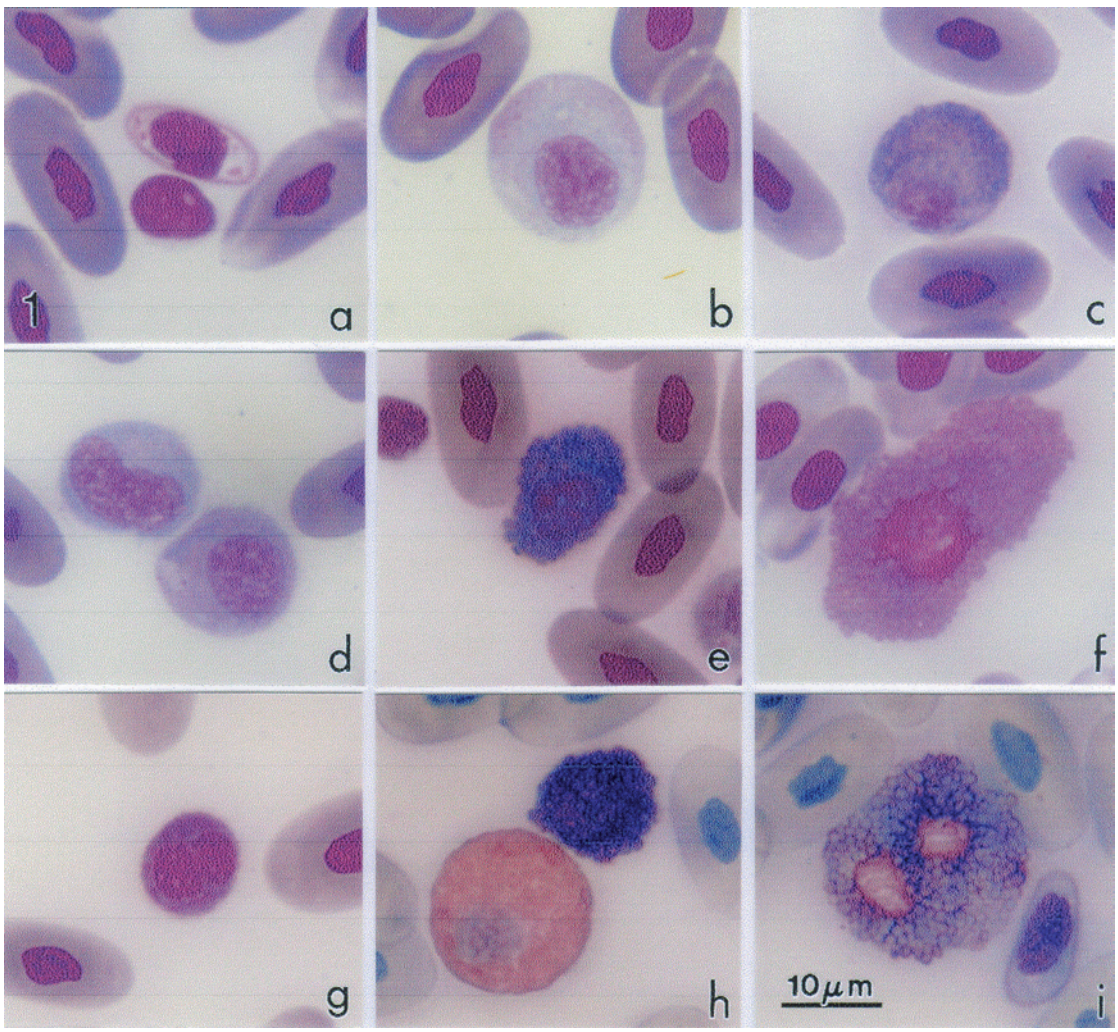

Figure 1 Blood cells in puff-faced watersnakes (a) a lymphocyte (lower cell) and a thrombocyte (b) an azurophil, (c) a heterophil, (d) a monocyte (left cell) and an azurophil, (e) an eosinophil, (f) a large eosinophil, (g) a basophil, Wright-Giemsa stain, (h) a 17 μm heterophil and an 14 μm eosinophil. Wright's stain, (i) a large eosinophil, Wright's stain.

parasitophorous vacuole membrane (Figure 3b).

DISCUSSION

The incidence of hematozoa infection in puff-faced watersnakes were as high as the other snakes in Queen Saovabha Memorial Institute (Salakij *et al*., 2001). Some hematological values were different from the normal hematologic parameters for reptile (Mader, 2000) such as the PCV was lower whist the total WBC and the plasma protein was higher than the reference (Mader, 2000). This study also revealed that hematozoa parasitism of puff-faced watersnakes erythrocytes had no effect on anemia since there was no significant difference of all erythrocyte parameters. These results support the finding that no clinical disease was demonstrated in parasitized snakes (Campbell, 1986).

Lymphocytes in puff-faced watersnakes were the most prevalent circulating cells like those in King cobra (Salakij *et al*., 2002) and the other snakes (Mader, 2000). Some researchers characterize azurophils as monocytes with azurophilic granules (Campbell, 1986). The finding of phagocytosing trypanosome by azurophil in TEM suggested that they may play a major role in elimination of trypanosome. This finding was observed only by TEM may because of the close

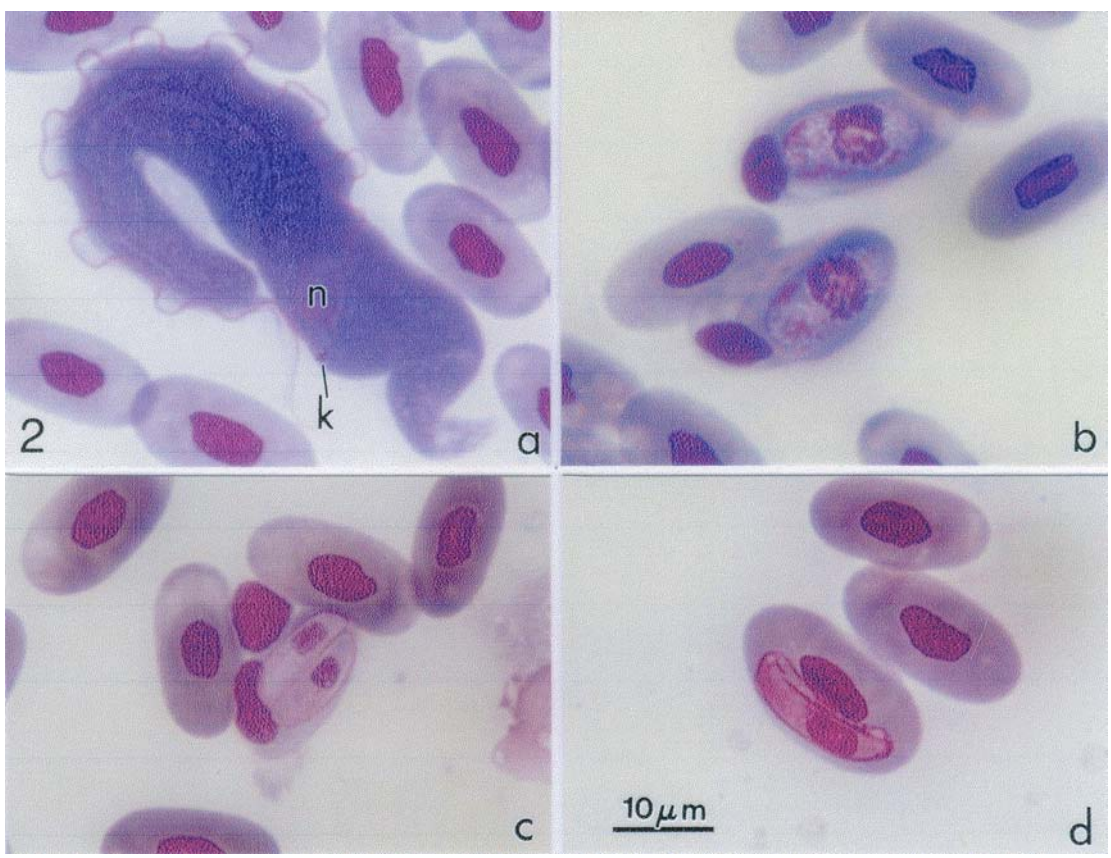

Figure 2 Hematozoa found in puff-faced watersnakes (a) trypomastigote of *Trypanosoma* sp. showing nucleus (n) and kinetoplast (k), (b) gamont of *Haemogregarina* sp, (c) two small gamonts of *Hepatozoon* sp., Note the displacement of the erythrocyte nucleus (d) large and curve gamont of *Hepatozoon* sp., Wright-Giemsa stain.

contact of the azurophils and the trypanosomes in the buffy coat before they were fixed with glutaraldehyde.

It is difficult to differentiate eosinophils from basophils in WG stained smears because of the bluish coloration of their granules. They were identifed more easily on Wright's stained preparation. The eosinophil granule characteristic in puff-faced watersnakes was similar to those of iguanas and psittacines (Hawkey and Dannett, 1989) which they contained dark purple staining metachromatic granules that obscure the unlobed nucleus. The large-sized eosinophils found in puff-faced watersnakes should be the characteristic of eosinophil in snakes which were larger than those of the other reptiles (Mader, 2000).

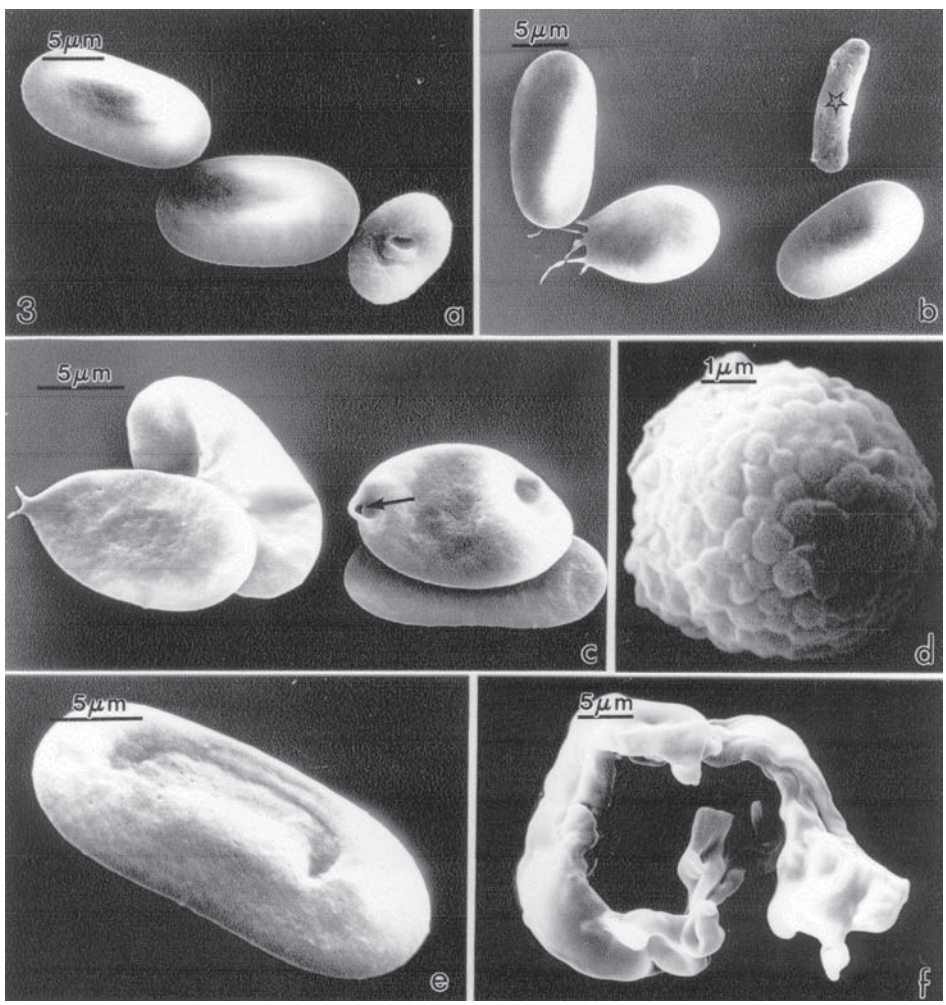

Figure 3 Scanning electron photomicrographs of (a) two normal erythrocytes and a thrombocyte, (b) euglenoil-shaped erythrocyte and parasitophorous vacuole membrane containing gamont of *Hapatozoon* sp. (star), (c) abnormal erythrocytes showing double appendages and cytoplasmic hole (arrow), (d) an eosinophil showing custard apple-like appearance of granule contour, (e) an enlarged erythrocyte containing *Hepatozoon* gamont, (f) a cluster of trypanosomes.

The high number of eosinophils in the negative and positive groups (Table 2) may be influenced by parasitic stimuli or other stimuli (Mader, 2000). The finding of eosinophils in puff-faced watersnake confirm the existence of these leukocytes in snakes eventhough they were not identified in eastern diamondback rattlesnakes (Alleman *et al*., 1999).

Trypomastigote form of trypanosomes in puff-faced watersnakes was different from *Trypanosoma hydrae* in broad-band watersnake from Louisiana (Chia and Miller, 1984). Trypanosomes were detected only in puff-faced and rainbow watersnakes of the QSMI (Salakij *et al*., 2001).

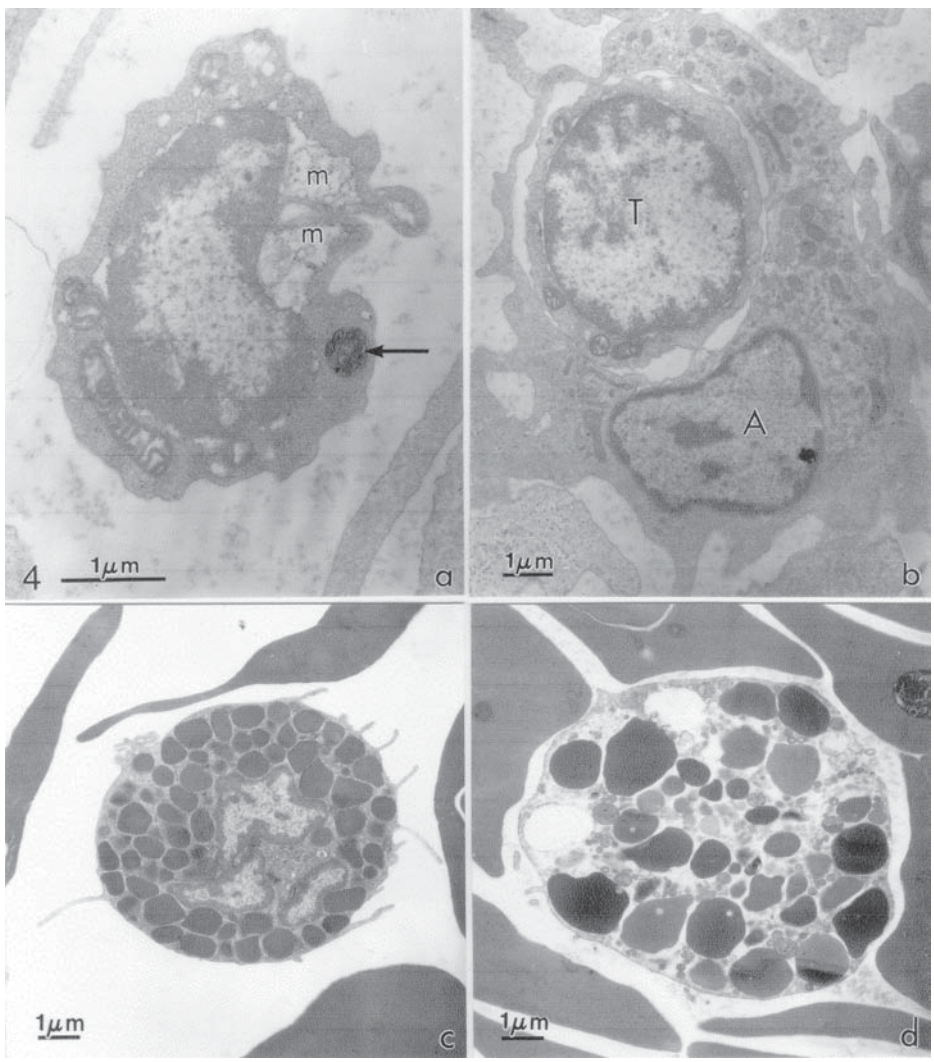

Figure 4 Transmission electron photomicrographs of (a) cross-section of a trypanosome containing nucleus, mitochondria, multivesicular bodies (m) and dense granular granule (arrow), (b) an azurophil (A) pseudopodia is surrounding a trypanosome (T), (c) an eosinophil with homogeneous granules and cytoplasmic process, (d) a heterophil with vacuoles and heterogeneous electron density granules.

The small gamonts of *Hepatozoon* sp. were similar to those found in the banded krait (*Bangarus fasciatus*) of the QSMI (Salakij *et al*., 2001). The large gamonts found in puff-faced watersnakes were referred as medium-sized gamonts when compared with the larger gamonts found in mangrove snakes and mangrove pit vipers of the QSMI (Salakij *et al*., 2001). *Haemogregarina* sp. was found not only in watersnakes but also in Burmese python, mangrove snakes and rainbow watersnakes of the QSMI (Salakij *et al*., 2001). These three kinds of snakes were not fed on puff-faced watersnakes so they were not transmitted by eating.

This study provides more information on the hematology, morphology and ultrastructure of blood cells in puff-faced watersnakes. This may be benificial for further study and related research.

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