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Hemolivia mauritanica (Haemogregarinidae: Apicomplexa) infection in the tortoise *Testudo graeca* in the Near East with data on sporogonous development in the tick vector *Hyalomna aegyptium*

PAPERNA I.*

Summary:

Testudo graeca tortoises were collected in the northern and southern Golan Heights (Israeli occupied territory of south Syria), and various locations in Israel and Palestine. *Hyalomma aegyptium* ticks were found only on Golan Height tortoises, and only the tortoises and ticks from the northern Golan Heights were infected with *Hemolivia mauritanica*. Tortoises became infected after ingesting infected ticks. Male ticks carrying sporocysts, which remain attached to tortoises for extended durations, apparently served as the source for dissemination of new infections among tortoises. Sporogenesis followed the pattern observed in the two other known species of *Hemolivia*, though there was some evident variation in fine-structural detail. The sutural slit detected in the *H. mauritanica* mature sporocyst wall was reminiscent of the suture characteristic of Coccidia of heterothermic vertebrate hosts; it could be a common ancestral character for both hemogregarines and Coccidia.

KEY WORDS : *Hemolivia mauritanica, Hyalomma aegyptium, Testudo graeca,* Near-East distribution, sporogenesis, ultrastructure.

Résumé : Infection de la tortue *Testudo graeca* au Proche-Orient par *Hemolivia mauritanica* (Haemogregarinidae : Apicomplexa) avec informations sur la sporogenese chez la tique vectrice *Hyalomna aegyptium*

Des spécimens de la tortue Testudo graeca *ont été collectés dans les parties nord et sud des hauteurs du Golan, et en divers endroits d'Israel et de Palestine. Des tiques de l'espèce* Hyalomna aegyptium *furent trouvées sur les tortues des hauteurs du Golan, mais seules les tiques et tortues de la partie nord étaient infectées par* Hemolivia mauritanica. *L'infection des tortues était consécutive à l'ingestion de tiques. Des tiques mâles, porteuses de sporocystes, et fixées aux tortues pendant de longues périodes, étaient apparemment la source de dissémination de nouvelles infections parmi les tortues. La sporogenèse, conforme à celle décrite chez les deux autres espèces connues du genre* Hemolivia, *présentait cependant quelques différences au niveau ultrastructural. L'arête suturale du sporocyste mature d'*H. mauritanica *évoquait la suture caractéristique des coccidies d'hôtes vertébrés hétérothermes ; cette ressemblance suggère qu'il s'agit d'un caractère ancestral commun aux hémogrégarines et aux coccidies.*

MOTS CLÉS : Hemolivia mauritanica, Hyalomma aegyptium, Testudo graeca, *Proche-Orient, sporogenesis, ultrastructure.*

INTRODUCTION

Hemolivia mauritanica (Sergent & Sergent, 1904) Landau & Paperna, 1997 was first described from an Algerian specimen of *Testudo graeca* L. Brumpt (1938) demonstrated that the sporogonous stages seen by Laveran & Negre (1905) in the tortoise tick *Hyalomma aegyptium* L. are infective by ingestion to *T. graeca*.

Recently, new records have been published on the distribution of *H. mauritanica* infections among *T. graeca* in Bulgaria and Turkey, and *T. marginata* in Greece, while *T. hermanni* from Bulgaria, Greece and Croatia were found negative (Široký *et al.*, 2005); both tortoise species were infested with *Hy. aegyptium*, and sporocysts were recovered from ticks removed from both hosts (Široký *et al.*, 2005).

Landau & Paperna (1997) recognized the generic relationship between this tortoise hemogregarine and *H. stellata* from *Bufo marinus* (Petit *et al.*, 1990) and *H. mariae* from *Tiliqua rugosa* (Smallridge & Paperna, 1997). The most conspicuous common characters were the oogonous-sporogonous development in the gut epithelium of ixodiid ticks, the stellate or triangular oocysts and a motile stage, progeny of oocysts — the sporokinete, which precedes differentiation of the encysted sporocyst. These features were confirmed by ultrastructural studies of *H. mariae* (Smallridge & Paperna, 2000a,b) and *H. stellata* (Boulard *et al.*, 2001). Our study of apicomplexan parasites of reptiles in the Near East included terrapins (Paperna, 1989) and tortoises. In this communication, we report our findings and laboratory studies on *H. mauritanica* infections in the land tortoise *T. graeca* and their tick vector *Hy. aegyptium*.

MATERIALS AND METHODS

Studied tortoises, *Testudo graeca*, were collected from the following locations (grids of 1978 edition of map of Israel):

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1 - Shoham (outskirts of Tel Aviv near Ben Gurion Airport), Israel (162N/143E) - one adult female, May 2, 2000.
2 - Near Kisra on the eastern sloped of the Samaria mountains, Palestine, at an elevation of ~ 600 m above sea level (166N/184E): two adult females, one adult and one juvenile male, and a very young specimen, May 2, 2000.
3 - Various locations (unrecorded) in the coastal plain, Israel: five adult females and three adult males, March 8, 1999.
4 - Masáda Oak forest, northern Golan Heights (Israeli occupied territory of south Syria, 292 N/221 E), elevation 1,025 m above sea level: five adults (sex was not recorded). Tortoises were infested with *Hy. aegyptium*, more ticks were also collected from other tortoises left in the habitat, May 20, 2000
5 - Kanaf settlement, southern Golan Heights, east of Lake Kinneret (252 N/215 E), elevation of 360 m above the lake (150 m above sea level): 10 adults, sex not recorded, infested with ticks, during May, 2001.
Tortoises were bled by piercing the leg or tail with a needle. Blood films or dots were air-dried, fixed in methanol and stained for 1 h in Giemsa (15 % in pH 7.4 phosphate buffer). Isolated ticks were kept in cotton-plugged vials at the ambient temperature of 21 to 30° C.
For transmission electron microscopy (TEM), ticks (two males, two non-engorged females and one engorged nymph) removed from infected tortoises (from site 4) were dissected in the fixative 2.5 % glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4). Infection was verified by scanning for released sporocysts in the sediment. Dissected portions of the gut were left in the fixative for 24 h at 4° C, then rinsed repeatedly in the same buffer, post-fixed in 1.0 % osmium tetroxide in the same buffer for 1 h and, after rinsing in the same buffer, dehydrated in graded alcohols and embedded in Agar 100® resin (Agar Scientific Ltd). Thin sections, cut with a diamond knife on a Reichert Ultracut ultratome, were stained on grids with uranyl acetate and lead citrate and examined in a JEOL 100CX TEM.

RESULTS

Infection in tortoise

H. mauritanica infection was detected only in the blood of the five tortoises from Masáda forest (Fig. 1). Infection was detected from May 20, 2000; by September 9 and October 18, 2000, high infection was still found in one of the five tortoises, and in one slide an intraerythrocytic dividing meront was observed (Fig. 2). Intraerythrocytic meronts readily occur in *Haemogregarina* spp. infections of terrapines (Paperna, 1989).

Infection in ticks

Adult male (seven) and non-engorged female (six) *Hy. aegyptium* ticks were removed from tortoises collected in Masáda forest. When dissected, all but one male and one non-engorged female, were found to have variable numbers of sporozoite-loaded sporocysts. Sporozoite-loaded sporocysts were also found in an engorged nymph. The live sporocysts were oval, 25-28 × 12-15 µm in size, and contained 16 to 18, 15 × 12 × 5 µm in size sporozoites (Figs 3, 4). Residuum was seen in some sporocysts (Fig. 4).
None of the engorged and non-engorged male or female ticks (total 10) removed from the tortoises collected near Kanaf settlement (site 5) were found to contain hemogregarine sporocysts.

Experimental transmission from tick to tortoise

A young, infection-free tortoise from site 3 was fed by May 30, 2000 on the abdominal contents of four, low-to-moderately sporocyst-infected adult male ticks. Blood examined June 28, 2000 revealed one intraerythrocytic meront (Fig. 2) and by August 9, 2000 it revealed moderate infection of *H. mauritanica* gametocytes.

Fate of tick infestation in laboratory held tortoise

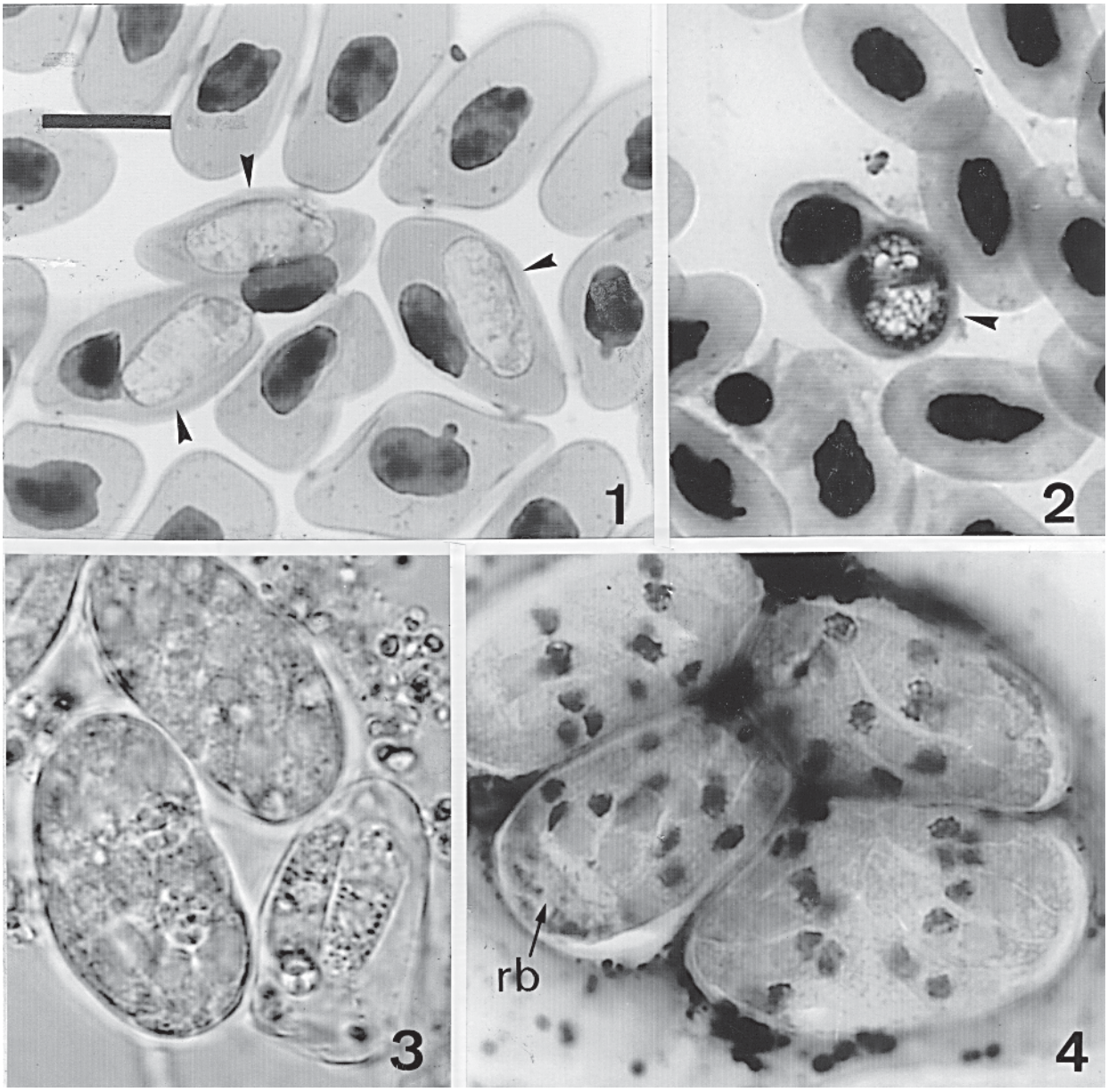
Tick infestation of the Masáda tortoises kept in the laboratory gradually disappeared: females failed to become engorged and dislodged first, while males remained, the last one untill October 18, 2000. The last remaining male ticks still contained sporocysts with intact sporozoites.
On the May 30, 2000 one partly engorged female laid some eggs; after storage at 16° C, eggs were returned to ambient temperature (20-31° C) on September 9, 2000 and hatched 21 days later. Two tortoises from site 2 and two from site 4 were exposed to tick larvae repeatedly on October 7, October 11, and October 14. Larvae failed to become established on the tortoises. The same results were obtained with tick larvae raised from another batch of eggs removed from refrigeration on the October 14, and hatched at the ambient temperature of 26 to 31° C on October 28, 2000 (after 14 days).

Electron microscopic study of sporogonic developmental stages in the tick

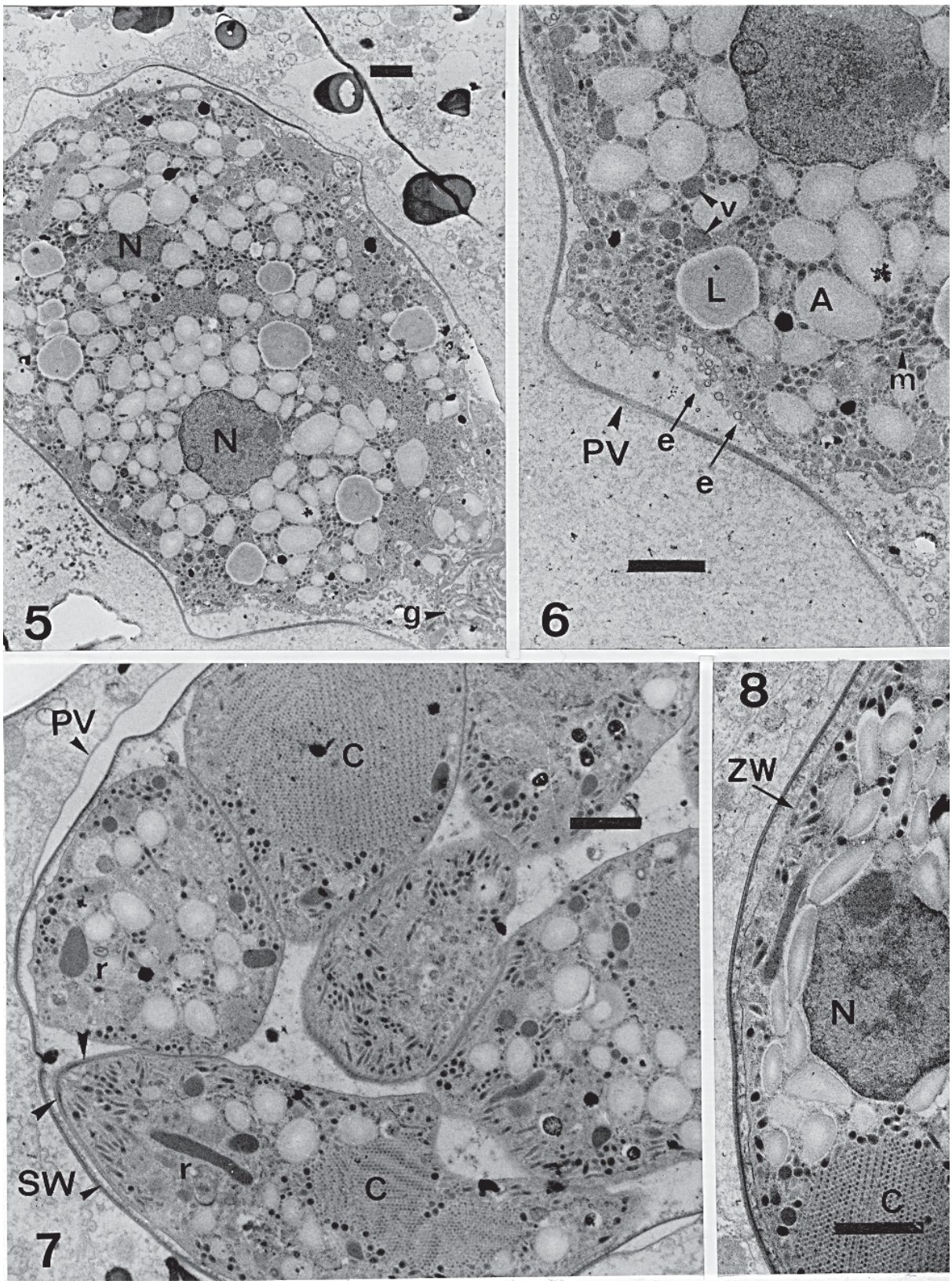
Infected ticks revealed only sporocyst stages. Sporocysts developed inside gut cells within a parasitophorous vacuole (PV) (Fig. 5). The wall of the PV was bila-

minated, enclosing an inner electron-dense lining. The non-differentiated early sporocyst's boundary contained shallow and deep invaginations, and was allied on the PV side with many even-sized round vesicles (Fig. 6). The cytoplasm was dotted with electron-dense granules, filled with micronemes and small electron-dense vesicles (Fig. 6), contained many amylopectin bodies, a few lipid vacuoles, two bodies of nucleoplasm, and a large golgi apparatus (Fig. 5). Two projections filled with micronemes appeared on the sporocyst surface, reminiscent of apical complex structure (not shown). Towards the completion of sporocyst differentiation, the host cell gradually degenerated into a vesicular sac, leaving the sporocyst to float in a large intercellular space.

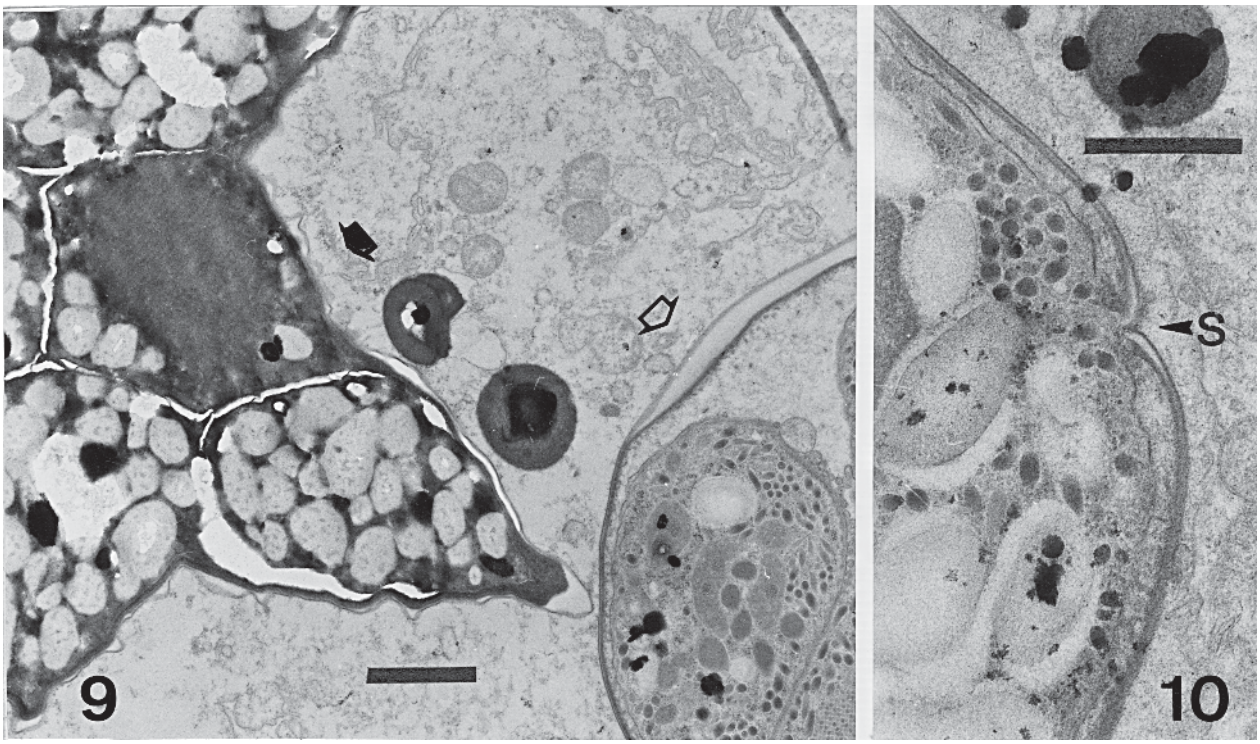
Sporocysts differentiated into sporozoites remained enclosed within a PV. The formed sporozoites replaced the sporocyst cytoplasm, but remained invested within a double-layered sporocyst wall which further thickened by deposition of electron-dense substance (Fig. 7). The sporozoites exhibited the characteristic apicomplexan formations. The sporozoite was initially bound by a single-layered wall (Fig. 8) which later consolidated into a pellicle. At the base of the apical complex, the pellicle formed a thickened ring (Fig. 7). The rhoptries and micronemes spread throughout the length of



Figs 1-4. – Fig. 1. *Hemolivia mauritanica* gametocytes (arrowheads) in blood film from *Testudo graeca*, scale bar = 10 µm. Fig. 2. Schizont of *Hemolivia mauritanica* in blood film from *Testudo graeca*. Fig. 3. Live sporocysts of *Hemolivia mauritanica* from gut squash of *Hyalomma aegyptium*. Fig. 4. Same sporocysts fixed and stained in Giemsa (rb – residual body; Figs 1-4 same magnification).



Figs 5-8. – Electron micrographs of sporocysts and sporozoites of *Hemolivia mauritanica* from gut contents of *Hyalomma aegyptium*, scale bar = 1 µm. Fig. 5. Premature sporocyst (g – golgi apparatus, N – nucleus). Fig. 6. Magnified view of the sporocyst invaginated and vesiculated wall (e), A – amylopectine granules, L – lipid vacuole, m – micronemes, PV – parasitophorus vacuole, v – electron-dense vesicles. Fig. 7. Sporocyst differentiated to sporozoites (C – crystalline body, r – rhoptries, SW – sporocyst wall, arrowhead – thickened pellicular ring). Fig. 8. Magnified view of a sporozoite (ZW – unilayered sporozoite wall).



Figs 9-10. – Electron micrographs of *Hemolivia mauritanica* sporocysts, scale bar = 1 µm. Fig. 9. Early (open arrow) and ripened (dark arrow) sporocysts. Fig. 10. View of the sutural slit (S) on the sporocyst wall.

the sporozoite. The other conspicuous organelles were the crystalline body and the amylopectin bodies. The nucleus and golgi complex were noticeable in some merozoites. Further thickening of the sporocyst wall rendered the sporocyst increasingly non-permeable, consequently demonstrating increased processing distortion (Fig. 9). The sporocyst wall was interrupted by a distinct sutural slit (Fig. 10).

DISCUSSION

The very limited data on the distribution of the tortoise *Hemolivia* in the levant suggests that the southern limit of the distribution range of this parasite is in southern Syria. Infection present in the northern Golan Heights (Masáda) and is apparently absent further south, in the Golan Heights around Lake Kinneret (Kanaf) as well as further south in Israel and Palestine. The northern Golan Heights with its higher altitude (~ 1,000 m), lush vegetation (oak forest at the site of collection), lower temperatures (~ 0° in winter) and heavier precipitation (~ 1,000 mm annually) is more comparable to the northern Mediterranean environment from which many of the infection in tortoises have been reported (Široký *et al.*, 2005). The more southern habitats (of ~ 600 m altitude) are more arid (~ 400 mm precipitation annually) with sparser and lower vegetation (Climate in Israel: http://www.tapuz.co.il). The environmental background of the infected tortoises found by Sergent & Sergent (1904) in Algeria was not elaborated upon. According to Hoogstraal (1956) the tortoise *Hyalomma* occurs in the European, Near Eastern Mediterranean and Black Sea regions. It seems, however, that the vector tick, *H. aegyptium* has a narrower distribution range than the tortoises and a wider range than *H. mauritanica*.

No explanation can be offered to the failure of attached female ticks to become engorged, or the refusal of larvae to attach to tortoises. *Hy. aegyptium* might be more requisite in its demand to ambient temperatures and humidity, which could also explain the limits of its natural range.

The haemogregarine genus *Hemolivia* has representatives additional to tortoises (*H. mauritanica*, Michel, 1973, Landau & Paperna, 1997) in lizards (*H. mariae*, Smallridge & Paperna, 1997) and in toads (*H. stellata*, Petit *et al.*, 1990). All of *Hemolivia* vectors are ixodiid ticks. However, ticks also vector other hemogregarinids – *Hepatozoon* spp. of reptiles and mammals. What is unique for *Hemolivia* is its biological development in the tick: oogony and sporogony which take place in the tick's gut epithelial cells and the stellate (or irregular, in tortoise *Hemolivia*) oocysts which gives a progeny of mobile sporokinetes (Petit *et al.*, 1990; Landau & Paperna, 1997; Smallridge & Paperna 1997). The

entire sporogonic development of the dog *Hepatozoon* (*H. americanum*), also transmitted by an ixodiid tick (*Amblyoma maculatum*), appears to occur similarly within the gut cells of its tick host (*Mathew et al.*, 1999). For better insight into the relationship between canine *Hepatozoon* and *Hemolivia*, this study, as well as earlier studies on the development of *H. canis* in its tick host *Rhipicephalus sanguineus* by Wenyon (1911) and Christophers (1912), should be confirmed by ultrastructural studies.

Hepatozoon kisrae Paperna, Kremer-Mecabell & Finkelman 2002 infecting *Agama stellio* is also transmitted by *Hy* cf. *aegyptium* (the vector population peferably feeds on *A. stellio,* Paperna *et al.*, 2002); however, its development in the tick, similar to reptile *Hepatozoon* spp. transmitted by mosquitoes (Smith & Desser, 1997) takes place in the haemocoel (Paperna *et al.*, 2002). Transmission to tortoises is *via* ingestion of infected ticks. Male ticks that remain attached to tortoises until the end of the summer are also heavy infected with sporocysts and are likely to be the vehicle for perpetuating infection into the next year.

The sporogenesis process follows the pattern observed in the other two species of *Hemolivia* (Smallridge & Paperna, 2000b; Boulard *et al.*, 2001), although there is some evident variation in fine-structural details: *H. mauritanicum* early sporocysts lack the crystalline bodies and the sphere-filled vacuoles prominent in *H. stellata*. The numerous micronemes and the apical projections seen in *H. mauritanicum* are absent in the latter two species. The invaginated border of the non-differentiated sporocyst, accompanied on its PV side by numerous round vesicles suggests an absorptive function, whereas this function in *H. mariae* seems to be fulfilled by the PV border (Smallridge & Paperna, 2000b).

The hardening of the mature sporocyst wall is a general phenomenon for all terrestrial-host hemogregarines – *Hemolivia* as well as *Hepatozoon* (Paperna *et al.*, 2002), although none of these are challenged to withstand desiccation in the course of their transmission (by the predation of the vector).

The sutural slit detected in the *H. mauritanicum* mature sporocyst wall has not yet been seen in the other species. It is reminiscent of the suture characteristic of Coccidia of heterothermic vertebrate hosts (Paperna, 1995, Jirkú *et al.*, 2002) it could be a common ancestral character of both hemogregarines and Coccidia.

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