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Endogenous development of *Hemolivia mauritanica* (Apicomplexa: Adeleina: Haemogregarinidae) in the marginated tortoise *Testudo marginata* (Reptilia: Testudinidae): evidence from experimental infection

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Abstract. Six young tortoises *Testudo marginata* Schoepff, 1792 were experimentally infected with *Hemolivia mauritanica* (Sergent et Sergent, 1904). The prepatent period ranged from 6 to 8 weeks. Young, smaller, club-like forms (6–9 × 3–6 µm) of gametocytes appeared in the peripheral blood first, whereas mature, elongated, cylindrical forms (9–12 × 5–7 µm) were detected after 1–2 weeks and predominated during later patency. Three of the infected tortoises were euthanized and dissected to study the endogenous stages. Meronts occurred in the cells of the reticulo-endothelial system and in the erythrocytes; these were observed mostly in parenchymatous organs. Mature forms measured 14.2 × 9.3 µm and contained 7–12 merozoites. Cysts with two (exceptionally one) cystozoites were also found predominantly in parenchymatous organs and measured 14.8 × 7.9 µm. Pathological changes attributable to *Hemolivia* were mild and limited to liver and kidneys. The role of individual developmental stages of haemogregarines is discussed with respect to evolution of heteroxenous life cycle and long-term persistence of parasites in their intermediate hosts.

The genus *Hemolivia* Petit, Landau, Baccam et Lainson, 1990 is defined as a haemogregarine of ectothermic vertebrates with presence of erythrocytic gamogony, and erythrocytic and extra-erythrocytic merogony and cystogony. Sporogony consisting of two phases occurs within gut epithelial cells of the definitive hosts, ixodid ticks, which serve as vectors. In the first phase, conjugation and fertilisation of macrogametes by microgametes occurs, followed by the formation of oocysts, which contain the motile sporokinetes (without formation of sporocysts). In the second phase, sporokinetes released from oocysts, penetrate new cells to produce sporocysts, later containing sporozoites, infective to intermediate vertebrate host (IH). There is no evidence of transovarian transmission within ticks (Petit et al. 1990).

The type species of the genus, *Hemolivia stellata* Petit, Landau, Baccam et Lainson, 1990, infects toads *Bufo marinus* (Linnaeus, 1758) and ticks *Amblyomma rotundatum* Koch, 1844. The genus currently contains two other species, *H. mariae* Smallridge et Paperna, 1997 affecting lizards *Tiliqua rugosa* (Gray, 1825) and ticks *Amblyomma limbatum* Neumann, 1899, and *H. mauritanica* (Sergent et Sergent, 1904) from the Palaearctic tortoises of the genus *Testudo* Linnaeus, 1758 and ticks *Hyalomma aegyptium* (Linnaeus, 1758) (Sergent and Sergent 1904, Petit et al. 1990, Smallridge and Paperna 1997).

Hemolivia mauritanica was originally described as *Haemogregarina mauritanica*, from an Algerian specimen of the spur-thighed tortoise *Testudo graeca* Linnaeus, 1758 (Sergent and Sergent 1904). Laveran and Nègre (1905) and Brumpt (1938) provided a description of its life cycle, developmental stages, and oral transmission via tortoises feeding on infected ticks. Michel (1973) on the basis of characters of the parasite's sporogony and merogony transferred it to the genus *Hepatozoon* Miller, 1908. Finally, Landau and Paperna (1997), after the reexamination of Brumpt's original material, revised the taxonomic state of the parasite, which is currently considered to be a member of the genus *Hemolivia*. Recently, we have found *H. mauritanica* in blood smears from wild, as well as from long-term captive, marginated tortoises *Testudo marginata* Schoepff, 1792 from Greece (Široký et al. 2004, 2005).

Despite the fact that more than hundred years have passed since *H. mauritanica* was first described, its life cycle, natural means of infection and mechanisms for long-term persistence in the host remain unknown. The goal of our study was to elucidate the endogenous development and morphology of the developmental stages of *H. mauritanica* in its newly recognized host, *T. marginata*.

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MATERIALS AND METHODS

Adult *Hyalomma aegyptium* from a laboratory breeding colony were allowed to feed on a haemogregarine-positive *T. marginata*. After their detachment, the ticks were dissected and examined for the presence of *Hemolivia* sporocysts. One infected tick was gently force-fed to each of six two-year-old haemogregarine-negative *T. marginata*. Blood was collected by puncture of the dorsal coccygeal vein once weekly. Blood smears were air-dried, fixed in absolute methanol and stained with Giemsa (diluted 1:10 in buffered water, pH 7) for 20 minutes, and examined with an Olympus Provis AX-70 microscope at × 1,000 magnification. During the prepatent period, the presence and morphology of both premature and mature gametocytes within the red blood cells (RBCs) were recorded. The size of infected RBCs was compared with those of uninfected cells. We counted only mature forms of erythrocytes; young polychromatophilic forms of RBCs were excluded from this analysis. Intensity of parasitaemia was counted as the number of infected RBCs for 10,000 cells.

Three tortoises having the highest level of parasitaemia were euthanized 10 (No. 1), 14 (No. 2) and 17 weeks (No. 3) post-infection, by overdosing with the barbiturate (Thiopental ICN), followed by decapitation. Lung, liver, kidney, spleen, brain, stomach, gut, heart, and skeletal muscle were sampled. Each organ was examined immediately as a fresh squash preparation and a portion was fixed in 10% buffered formaldehyde for histopathological examination. After the dehydration in a graded ethanol series, samples were embedded in paraffin, cut to 4 μm thickness and stained by standard haematoxylin-eosin procedure.

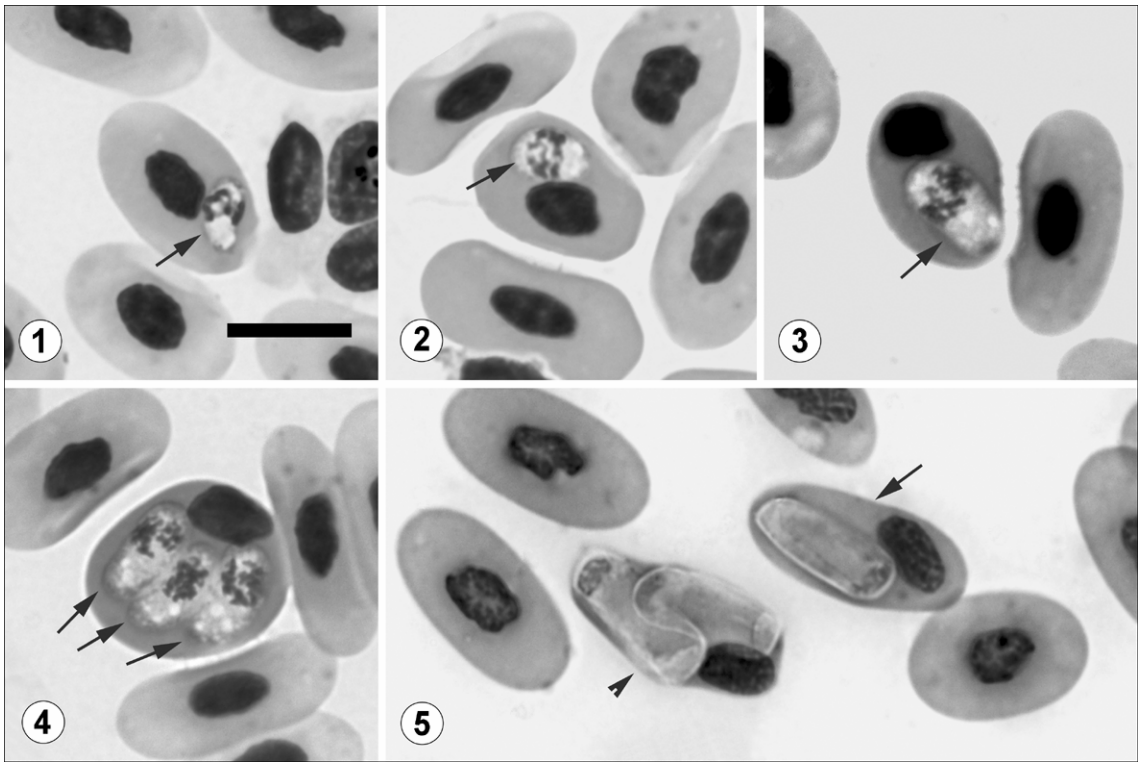
RESULTS

All six juveniles of *T. marginata* became infected with a prepatent period ranging from 6 to 8 weeks. Young forms of gametocytes (or merozoites sensu Smallridge and Bull 2001) appeared in the peripheral blood in the beginning of patent period; the mature forms appeared after 1–2 weeks and predominated later during the patency. In tortoise No. 1, 21.3% of the RBCs were infected 9 weeks post-infection, while in remaining tortoises (Nos. 2–5) parasitaemias of 5.9, 4.2, 1 and 0.8% infected RBCs respectively were recorded. In tortoise No. 6, only four infected RBCs per 10,000 cells were detected.

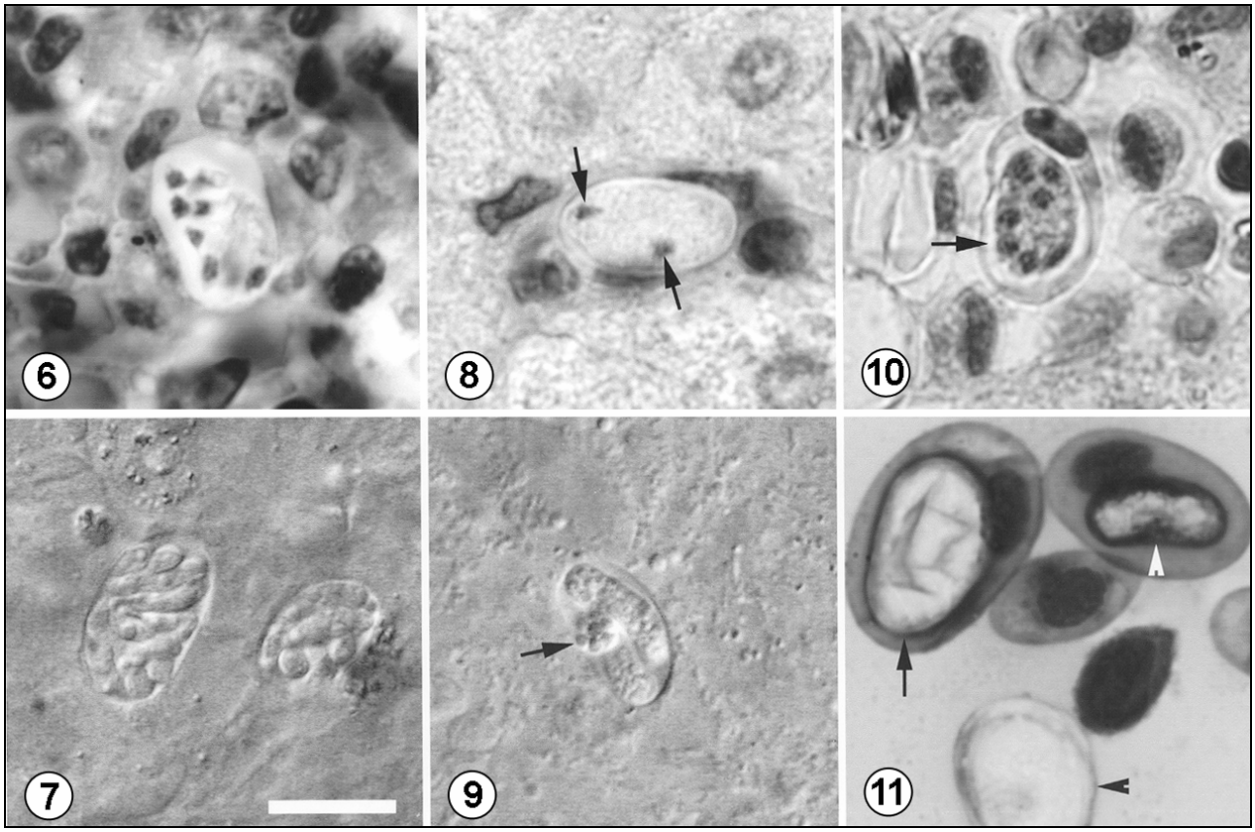
Developmental stages

Gametocytes in the peripheral blood Figs. 1–5

Gametocytes at different developmental stages were located solely in erythrocytes. The premature gametocytes were first small, club-like, 6–9 × 3–6 μm (n = 11; Fig. 1). Larger forms became more oblong, and meas-



Figs. 1–5. *Hemolivia mauritanica*, development of gametocytes within the erythrocytes of peripheral blood of *Testudo marginata*, tortoise No. 4. **Fig. 1.** Youngest club-like form marked by arrow, 49 days post-infection (DPI). **Fig. 2.** Premature oblong form of gametocyte (arrow), 49 DPI. **Fig. 3.** Premature gametocyte (arrow) with nucleus located sub-centrally, 49 DPI. **Fig. 4.** Erythrocyte infected by three premature gametocytes (arrows) simultaneously, 56 DPI. **Fig. 5.** Mature gametocyte with stain-resistant capsule and nucleus in polar position (arrow) and erythrocyte infected by two gametocytes (arrowhead), 165 DPI. Scale bar = 10 μm; all figures in the same scale.



Figs. 6–11. Meronts and cysts of *Hemolivia mauritanica* in histological sections (Figs. 6, 8, 10 – haematoxylin-eosin staining), unstained compressed slides (Figs. 7, 9 – Nomarski interference contrast), and blood smear stained with Giemsa (Fig. 11). **Fig. 6.** Meront in the spleen, tortoise No. 1, 70 DPI. **Fig. 7.** Two meronts in the liver of tortoise No. 1, 70 DPI. **Fig. 8.** Dizoic cyst in the liver, nuclei of cystozoites marked by arrows, tortoise No. 1, 70 DPI. **Fig. 9.** Dizoic cyst in the spleen, cyst residual body marked by an arrow, tortoise No. 3, 119 DPI. **Fig. 10.** Intraerythrocytic merogony, meront marked by an arrow, tortoise No. 1, 70 DPI. **Fig. 11.** Meront within the erythrocyte (arrow), free meront (black arrowhead) probably after destruction of erythrocyte, and premature gametocyte (white arrowhead), tortoise No. 1, 70 DPI. Scale bar = 10 μm; all figures in the same scale.

ured 7–10 × 4–6 μm (n = 12; Figs. 2–4), with their nucleus located sub-centrally. Mature gametocytes were elongated, oval to cylindrical, sometimes bean-shaped 9–12 × 5–7 μm (n = 22); they developed a stain-resistant capsule and nucleus located in a polar position (Fig. 5). Gametocytes affected the shape of infected RBCs to some degree. Uninfected erythrocytes measured 19.4 (17–24) × 10.7 (9–15) μm, shape index (SI, length/width) of 1.83 (1.38–2.33) (n = 30), whereas infected RBCs were shorter in average 17.1 (12–21) × 10.6 (8–15) μm, SI 1.63 (1.07–2.11) (n = 30) (Mann-Whitney test; length: $Z = 4.12$, $p << 0.01$; width: $Z = 0.76$, $p = 0.45$; SI: $Z = -13.08$, $p << 0.01$).

Meronts Figs. 6, 7, 10, 11

Merogony occurred in the cells of reticulo-endothelial system (Figs. 6, 7) and in the erythrocytes (Figs. 10, 11) primarily in the spleen, liver, kidneys and lungs. Meronts at different developmental stages were always observed within blood vessels and sinusoids. They were never seen in the parenchyma of internal organs. Primary and secondary meronts were not distinguished. The nuclei of premature meronts were arranged around the peripheral margin of the cytoplasm, and measured 11.3 (10–12) × 8.3 (6–9) μm (n = 13). Mature meronts measured 14.2 (13–17) × 9.3 (7–11) μm (n = 17) and contained 7–12 elongate merozoites, usually arranged in parallel within the long axis of meront.

Cysts Figs. 8, 9

These stages were found in the same locations as meronts, particularly in parenchymatous organs such as liver, spleen and kidneys. Interestingly, within the organs of specimen No. 3 examined 17 weeks post-infection the cystic stage predominated over the meronts. The cysts contained mostly two, exceptionally one, elongate, cucumber-shaped cystozoites, having a spongiform appearance in stained histological sections, whereas in fresh squash films they appeared finely granular with circular nucleus located sub-centrally. Spherical granules formed the residual body of the cyst (Fig. 9). Cysts were elongate, ellipsoidal to bean-shaped, measuring 14.8 (12–18) × 7.9 (6–10) μm in dizoic form (n = 26) and 13 × 7 (6–8) μm in case of rare monozoic cysts (n = 3).

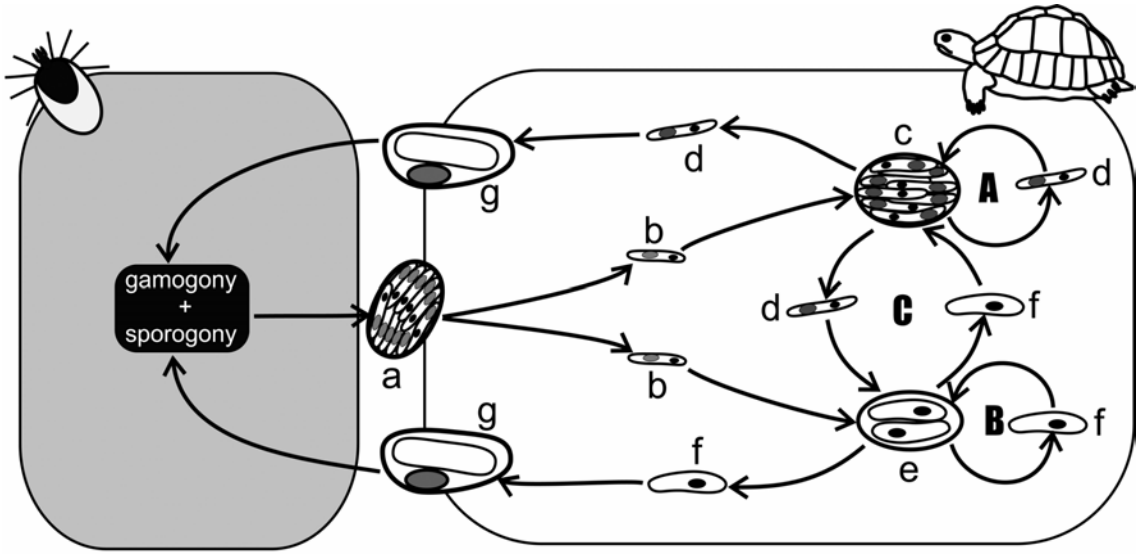


Fig. 12. Development of *Hemolivia mauritanica* in *Testudo marginata* shown in an open square area (development in the tick is not discussed, shown as shaded area). Sporocysts (a) located in tick are ingested by tortoise. Sporozoites (b) enter cells of the reticulo-endothelial system or erythrocytes, where they transform either to meronts (c) containing merozoites (d) or to cysts (e) with cystozoites (f). Gametocytes (g) develop in erythrocytes invaded either by merozoites or cystozoites. Three ways of long-term maintaining of infection are hypothesized: (**A**) cyclic merogony, (**B**) cyclic cystogony, and (**C**) initiation of merogony by cystozoites or conversely, initiation of cystogony by merozoites.

Pathological changes

Mild pathological changes attributable to *Hemolivia* were only observed in histological sections from the tortoise which exhibited the highest parasitaemia. These mild alterations were confined to the kidneys and liver and consisted of mild focal to multifocal lower nephron nephrosis and hepatocellular hydropic degeneration. Developmental stages of *Hemolivia* were observed within hepatic sinusoids; however, the hepatic parenchyma remained normal. The balance of the parenchymatous organs from all tortoises appeared normal, regardless of the degree of intraerythrocytic parasitism observed.

DISCUSSION

All experimentally inoculated tortoises became infected with *Hemolivia mauritanica*, and displayed a prepatent period range comparable with that observed in other *Hemolivia* species (Petit et al. 2000, Smallridge and Bull 2001). Except for the absence of so-called primary merozoites (sensu Smallridge and Bull 2001), the occurrence and morphology of other intraerythrocytic forms correspond to those described by Smallridge and Bull (2001) for *H. mariae* in Australian skinks. However, we observed the intraerythrocytic meronts only in the blood smears collected from the tortoise with the highest parasitaemia.

Among the haemogregarines parasitizing ectothermic hosts, the genus *Hepatozoon* has been the most frequently studied. The majority of authors reported on two types of meronts in *Hepatozoon*, usually called macro- and micromeronts, or Y meronts and X meronts, respectively (i.a. Smith 1996, Telford et al. 2001, 2002, Paperna et al. 2002). So far, no species of *Hemolivia* have been reported to share this trait (Michel 1973, Petit et al. 1990, Paperna and Smallridge 2001). Although we found meronts differing by numbers of merozoites or by size, we were unable to distinguish two distinct groups. Rather, they displayed a continuous range, with the extremes resembling the so-called macro- and micromeronts. Compared to Michel (1973), we observed both premature and mature meronts smaller in average, and containing fewer merozoites.

The role of cystic stages in the life cycle of haemogregarines is enigmatic. Landau et al. (1972) first described the presence of cysts in the intermediate host of *Hepatozoon domerguei*. Since that time, the cystic stages have been recorded in many *Hepatozoon* species (e.g. Paperna et al. 2002, Lainson et al. 2003, Paperna and Lainson 2004) and also in members of the genus *Hemolivia* (Michel 1973, Petit et al. 1990). Michel (1973) recorded cysts containing mostly two, but rarely also four or six cystozoites in *Hemolivia mauritanica*. The majority of cysts we observed were dizoic; monozoic forms were rare. Neither tetrazoic nor hexazoic forms were seen. The cysts we observed, particularly the monozoic forms, were smaller and more elongated than those reported by Michel (1973) in *Testudo graeca*.

Landau et al. (1972) suggested that the cystic stages were responsible for transmission of *Hepatozoon* between lizards (first IH) and a saurophagous snake (second IH). Smith (1996) concluded that many *Hepatozoon* species in snakes undergo a three-host life cycle, where

insectivorous frogs or lizards serve as the first IH, a snake predating on them is the second IH, and mosquitoes as definitive hosts that can feed on all – frogs (or lizards) and snakes. Subsequently, Smith and Desser (1998) described the life cycle of *Hepatozoon sipedon* with cystogony in frogs *Rana pipiens*, preceding the merogony in snakes *Nerodia sipedon* that were fed the liver of these frogs. Petit et al. (1990) suggested a complex role of cysts in oral transmission of *Hemolivia stellata* to toad, *Bufo marinus*. Such a "transmission hypothesis" fits well to haemogregarines with predatory intermediate hosts. However, the intermediate hosts of *H. mauritanica* are herbivorous tortoises. Thus, cysts of *H. mauritanica* either have some other function (see below) or represent an evolutionary relic stage inherited from a common ancestor. A second explanation suggests the transition from a three-host to a two-host life cycle. Recent studies focusing on the phylogeny of blood apicomplexans suggested their evolution from monoxenous apicomplexans of invertebrates (Barta 1989, Kopečná et al. 2006). Then, the ancient origin of two-host life cycle of *H. mauritanica* is more likely than secondary simplification from a more complicated three-host development.

With respect to the studies mentioned above, possible ways of endogenous development of *H. mauritanica* in tortoises are hypothesized in Fig. 12. Cystogony could serve as a parallel path to merogony and can represent an adaptation, responsible for long-term (perhaps even life-long) persistence of the infection. Our previous studies documented that *H. mauritanica* gametocytes can occur in a tortoises' circulating blood for at least 12 years (Široký et al., unpubl.). The lifespan of reptilian erythrocytes is definitely shorter (e.g. Davies and Johnston 2000) and gametocytes are considered to be the final stage of development in the vertebrate host. Therefore, long-term parasitaemia must be maintained somehow. The permanent presence of gametocytes can be explained several ways: (i) cyclic merogony, (ii) cyclic cystogony, or (iii) by consecutive release of dormant cystozoites or merozoites. Further, it is possible (iv) that dormant cystozoites can initiate another cycle of merogony after their activation.

Endogenous extra-erythrocytic development of haemosporidia is known to be responsible for inducing considerable pathology, both in birds and mammals. In contrast, haemogregarines are not considered to be particularly pathogenic (Desser 1993) and few studies deal with pathology caused by them. Reported changes include the alteration of erythrocytes' morphology and their overall shape, hypertrophy of cytoplasm or pycnotic and dislocated nuclei without pronounced clinical effect (Ball et al. 1969, Allison and Desser 1981, Siddall and Desser 1993). Meront-associated inflammatory lesions resulting in clinical disease were described by Wozniak et al. (1998). Pathology was observed in unnatural lizard hosts infected experimentally with *Hepatozoon* species that was transferred to them from naturally infected snakes (Wozniak and Telford 1991, Wozniak et al. 1996). In our study, only mild pathological changes were recorded in the kidneys and liver of the tortoise manifesting the highest parasitaemia. *Hemolivia mauritanica* infection does not appear to represent a serious threat for captive or wild tortoises of the genus *Testudo*.

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