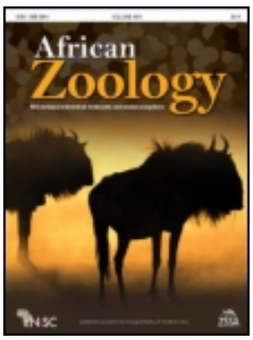

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First record of an intraleucocytic haemogregarine (Adeleorina: Haemogregarinidae) from South African tortoises of the species *Stigmochelys pardalis* (Cryptodira: Testudinidae)

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To date, four intraerythrocytic apicomplexans, namely the haemogregarines *Haemogregarina fitzsimonsi* and *Haemogregarina parvula*, and the haemoproteids *Haemoproteus testudinalis* and *Haemoproteus natalensis*, have been described from South African land tortoises. Recently, an intraleucocytic haemogregarine was observed in one species of tortoise, *Stigmochelys pardalis,* from the province of KwaZulu-Natal. Gamonts were identified in the monocytes and lymphocytes of 5/126 (4%) *S. pardalis*, but no additional stages were detected. Mixed infections with *H. fitzsimonsi* were observed for 2/5 (40%) of the parasitized *S. pardalis*, but the intraleucocytic gamont stages were larger than the intraerythrocytic gamont stages of both *H. fitzsimonsi* and *H. parvula*. The only other record of a chelonian intraleucocytic haemogregarine is of *Haemogregarina pseudemydis*, with stages described from the red and white blood cells of neotropical terrapins. Thus, the report of an intraleucocytic haemogregarine infecting a terrestrial tortoise from Africa is significant, although its taxonomic placement remains problematic at present.

Key words: intraleucocytic, haemogregarine, tortoise blood parasite, South African, apicomplexan taxonomy.

The biodiversity of tortoises in South Africa is high and includes 14 species/subspecies in five genera (Branch 2008). In the past six years, blood samples taken from tortoises from a number of sites across South Africa have been examined. This has resulted in the redescription of two haemogregarines, *Haemogregarina fitzsimonsi* Dias, 1953 and *Haemogregarina parvula* Dias, 1953 (see Cook *et al*. 2009), originally recorded from Mozambique, and of the haemoproteid *Haemoproteus testudinalis* (Laveran, 1905) Wenyon, 1915 originally reported from the Cape region of South Africa (see Cook *et al*. 2010). In addition, a new species of haemoproteid, *Haemoproteus natalensis* Cook, Smit & Davies, 2010 has been described (Cook *et al*. 2010). Overall, tortoises from five of the nine South African provinces have been examined by Cook *et al*. (2009, 2010), and those from KwaZulu-Natal (KZN) have the highest biodiversity of haematozoans, including all the above named parasite species, except for *H. testudinalis*.

In the present study, another apicomplexan haemogregarine was located in the blood of a tortoise species, *Stigmochelys pardalis*, from KZN, but this was intraleucocytic. Such intraleucocytic haemogregarines are rarely observed in chelonians, none apparently having been described previously from terrestrial tortoises (Levine 1988; Telford 2009). *Haemogregarina pseudemydis* Acholonu, 1974 appears to be the only other chelonian haematozoan described infecting leucocytes and was recorded from some 10 Neotropical terrapin species (Acholonu 1974; Levine 1988). Acholonu (1974) described intraerythrocytic trophozoite and gamont stages of *H. pseudemydis* and rare merozoite stages developing within leucocytes, the only stages recorded from cells of the white series, seemingly parasitizing only *Pseudemys floridana* (Le Conte, 1830), and likely transmitted by a leech vector (see Siddall 1995). The current paper is the first report of an intraleucocytic haemogregarine from terrestrial tortoises worldwide, unlike that of the above, in that ticks are more likely the vectors, suggesting the present study's haemogregarine to be of a completely different genus (see Široký *et al*. 2007; Cook *et al*. 2009).

For three years (2009–2011), 275 individual

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tortoises were studied, both wild (195) and captive (80), from four of nine provinces in South Africa. These provinces are Gauteng (GP), with tortoises examined from the Johannesburg Zoological Gardens and private collections within Johannesburg (both captive tortoise collections); KZN, with animals sampled from Mkuze Nature Reserve (27°39′0″S, 32°15′0″E) and Bonamanzi Private Reserve (28°3′42.1″S, 32°17′7.1″E) (both wild tortoise collections); the Northern Cape (NC), with tortoises sampled from Britstown, De Beers Diamond Route, Namaqualand and Tswalu Kalahari Private Reserve (all wild tortoise collections); and the Western Cape (WC), with animals examined from Arniston, De Hoop, De Mond, Elandsberg, Gouritzmond, Paarl, Paternoster, West Coast (all wild), and Paarl Butterfly Park (captive tortoise collections). Overall, eight indigenous tortoise species, in five genera, were studied, including: 99 angulate tortoises, *Chersina angulata* (Schweigger, 1812); 26 parrot-beaked padlopers, *Homopus areolatus* (Thunberg, 1787); three Bell's hinged tortoises, *Kinixys belliana belliana* Gray, 1830; seven Lobatse hinged tortoises, *Kinixys lobatsiana* (Power, 1927); two Natal hinged tortoises, *Kinixys natalensis* Hewitt, 1935; 10 Kalahari tent tortoises, *Psammobates oculiferus* (Kuhl, 1820); three Trimen's tent tortoises, *Psammobates tentorius trimeni* (Boulenger, 1886); and 126 leopard tortoises, *Stigmochelys pardalis* (Bell, 1828). Haematozoans were detected by collection of peripheral blood from the subcarapacial sinuses of host tortoises (see McArthur *et al*. 2004) (ethically approved by the Academic Ethics Committee of the Faculty of Science, University of Johannesburg, Reg. No. 920203595). The tortoises were then released in the wild or back into captivity. Thin blood smears were prepared, fixed in absolute methanol for 10 minutes and stained in Giemsa's stain (SIGMA) for 20 minutes; they were then screened with an Olympus CX21FS1 field light microscope (Olympus, Hamburg, Germany). Appropriate images were subsequently captured with a Zeiss Axiocam digital camera attached to a Zeiss Axioplan 2 photomicroscope (Carl Zeiss, Jena, Germany) equipped with a ×100 oil immersion objective, and measurements were taken as detailed in Cook *et al*. (2009). Results were compared with previous findings (Cook *et al*. 2009, 2010). Since molecular characterization of the intraleucocytic haemogregarine was unsuccessful at this stage, the method used will not be discussed in much detail. However, molecular description was attempted by fractionation of whole blood with a parasitaemia of 0.004% from an infected *S. pardalis*. The resulting buffy coat containing the leucocytes was collected, DNA extracted using a DNeasy Animal Tissue Kit (using the spin column protocol) (QIAGEN Ltd, U.K.), and parasite 18S rDNA amplified using two primer sets HEMO1/HEMO2 (Perkins & Keller 2001), apicomplexan and haemogregarine specific, and HEPF300/HEPR900 (Ujvari *et al*. 2004), *Hepatozoon* specific. Of the two primer sets, the HEPF300/HEPR900 primers were the only ones to produce a result, producing a band of ~600 bp. Unfortunately, no useable sequences were obtained.

Overall, haemogregarines were found parasitizing 40/275 (14.5%) of wild and captive tortoises. The intraleucocytic haemogregarine (Fig. 1A–G) was observed in only 5/275 (1.8%) of tortoises, all wild *S. pardalis*, and from a single province (KZN). Of the two previously observed haemogregarines (see Cook *et al*. 2009), *H. fitzsimonsi* (Fig. 1H,I) parasitized 36/275 (13.1%) of the present study's tortoises and *H. parvula* (Fig. 1H,I), 2/275 (0.7%). *H. fitzsimonsi* had a wide geographical range, occurring in tortoises from four provinces within the present study, GP, KZN, NC and WC. It was also observed in 5/8 (63%) of the tortoise species, namely *C. angulata, K. b. belliana, K. lobatsiana, K. natalensis,* and *S. pardalis.* Conversely, *H. parvula* apparently had a restricted range, and was recorded only from KZN, and only in 2/8 (25%) of the tortoise species, namely *K. b. belliana* and *S. pardalis*. These brief data on *H. fitzsimonsi* and *H. parvula* prevalence will be considered fully elsewhere (Cook *et al*. 2014). As for the haemoproteids, *H. testudinalis* and *H. natalensis* (see Cook *et al*. 2010), they were not detected during the present study.

Two of 13 (15.4%) individuals of *S. pardalis* from Mkuze Nature Reserve (KZN), and 3/5 (60%) *S. pardalis* from Bonamanzi Private Reserve (KZN) were found to be parasitized with the intraleucocytic haemogregarine. Gamont stages were seen parasitizing monocytes and lymphocytes (Fig. 1A–G), but no heterophils, eosinophils or thrombocytes were found to be affected. On average 0.004 ± 0.004 (0.001–0.01)% of total leucocytes were parasitized. In Giemsa-stained blood films gamonts sometimes appeared faintly recurved within monocytes (Fig. 1A), but mostly they appeared globular within these cells (Fig. 1B–E), and in lymphocytes (Fig. 1F,G), perhaps because they were tightly bound within a constraining para-

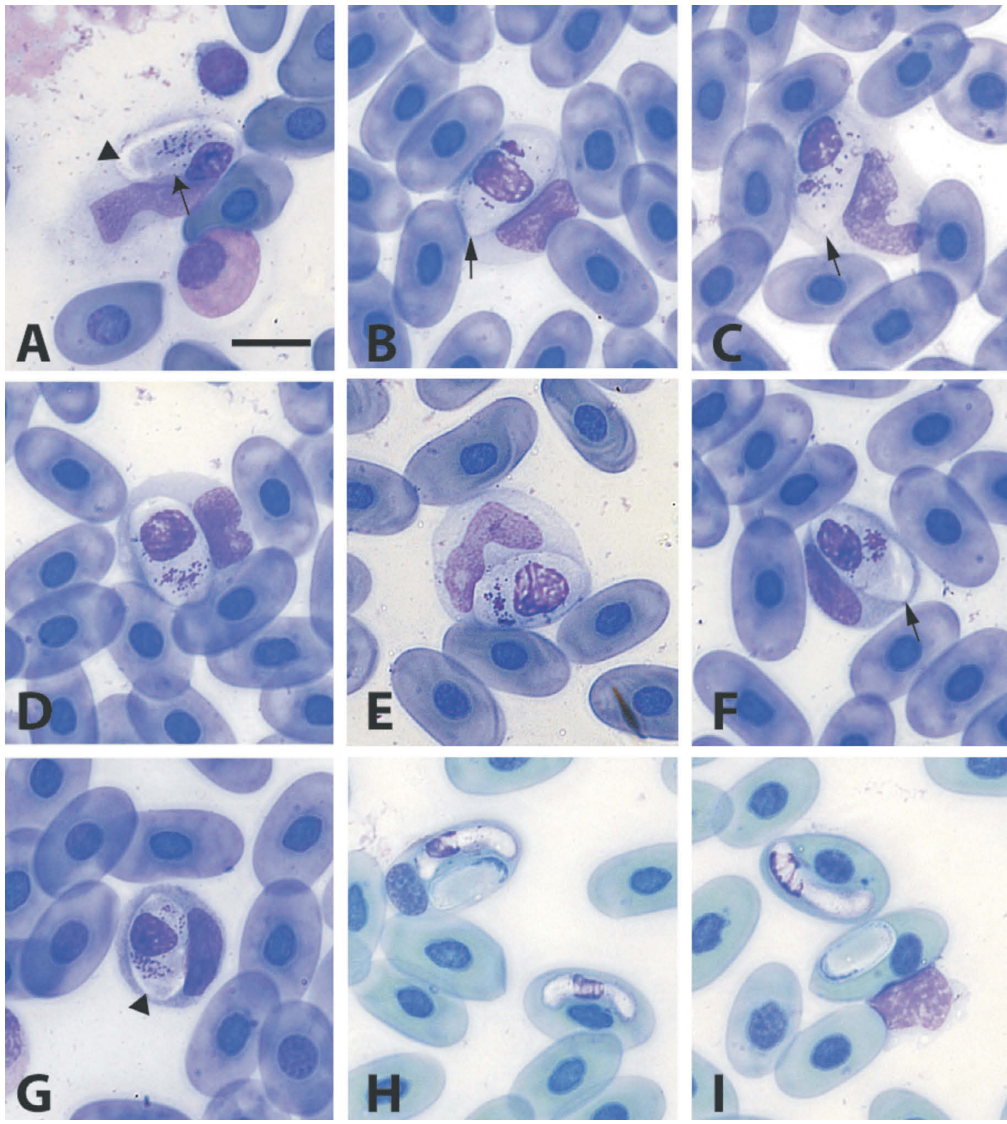

Fig. 1. Light micrographs of Giemsa-stained blood films showing the haemogregarine species observed in South African tortoises. **A–G**: Intraleucocytic haemogregarine from *Stigmochelys pardalis* from Mkuze Nature Reserve and Bonamanzi Private Reserve, KZN: **A–E**, parasitizing monocytes; **A**, arrow illustrating faintly recurved gamont, arrowhead indicating parasitophorous vacuole; **B**, **C**, arrows illustrating tapered (posterior?) pole of gamonts; **D**, **E**, gamonts with rounded poles; **F**, **G**, parasitizing lymphocytes; **F**, arrow indicating tapered (posterior?) pole of gamont; **G**, gamont with rounded poles, arrowhead indicates narrow parasitophous vacuole. **H**, **I**, Concurrent parasitism of intraerythrocytic *Haemogregarina fitzsimonsi* Dias, 1953 (slender form) and *Haemogregarina parvula* Dias, 1953 (globular, encapsulated form) in *Kinixys belliana belliana* from KZN. Note size differences among the haemogregarines. Scale bar = 10 µm.

sitophorous vacuole, which was visible only occasionally (Fig. 1A,G). In some gamonts one pole (anterior) was slightly broader and rounder than the other, which was tapered (posterior) (Fig. 1B,C,F). Gamonts measured 17.6 ± 0.9 (15.9–19.2) μm long, and 9.7 ± 0.8 (8–10.6) μm wide (n =11), with a surface area of 141.8 ± 12.5 (112.9–151.2) μm^2 (n =11). Presumed anterior to mid-nucleus measurements were 3.6 ± 1.5 (1.4–6.3) μm, and mid-nucleus to posterior measurements, 7.2 ± 1.6 (4.8–10.3) μm (n =11). Nuclear lengths and widths were 7.5 ± 1 (6–9) μm and

6.5 ± 1.2 (5.3–8.5) μm (n =11), respectively. Nuclei were deep-stained purple, oval, rounded or square in outline, and either dense or foamy in appearance. Deep red stained granules formed clusters in the generally grey-blue stained cytoplasm, especially in the vicinity of the nucleus (Fig. 1B–G), but also at some distance from it. Cytoplasm was occasionally vacuolated. Mixed infections were observed only with *H. fitzsimonsi*, and these occurred in 2/5 (40%) of the *S. pardalis* parasitized by the intraleucocytic haemogregarine (one from Mkuze Nature Reserve, the other from Bonamanzi Private Reserve).

The taxonomy of the intraleucocytic organism is problematic because to date it has not been possible to extract and amplify sufficient DNA from it to allow its sequencing (Cook *et al*. unpubl. data), possibly owing to the low parasitaemias encountered.

In comparison to *H. pseudemydis*, the only other chelonian haemogregarine with reported intraleucocytic stages is a terrapin (Acholonu 1974). It developed intraerythrocytic U-shaped trophozoites, apparently measuring 31.7 × 5.9 μm, and the developed trophozoites, looped with fused arms, were 13.6 × 4.2 μm. Shorter, bean-shaped intraerythrocytic gamonts measure 14 × 6 μm with a nucleus of 5.1 × 3.2 μm, and longer, slender, extracellular forms measure 17.5 × 3.7 μm with a nucleus of 8.4 μm in length. Meronts of *H. pseudemydis* are apparently intraleucocytic, spherical to oval and measure on average 10.9 × 9.7 μm (Acholonu 1974). The intraleucocytic organism described here, measuring 17.6 × 9.7 μm overall with a nucleus of 7.5 × 6.5 μm, is therefore much larger than the gamont and meront stages of *H. pseudemydis*, and more closely resembles its larger intraerythrocytic trophozoite stages. However, taking into consideration parasite stage dimensions of both *Haemogregarina stepanowi* Danilewsky, 1885 and *Haemogregarina macrochelysi* Telford, Norton, Moler & Jensen, 2009, well described haemogregarines of terrapins (see Telford 2009), which have similar U-shaped intraerythrocytic forms to *H. pseudemydis*, but are identified as gamont stages, suggesting that the largest stages in *H. pseudemydis* are also likely gamonts and are not trophozoites.

It has been considered that the parasite may be a stage in the development of *H. fitzsimonsi*, as this study demonstrated the haemogregarines occurring concurrently in some *S. pardalis* from KwaZulu-Natal. If the former is a stage of the latter, however, it begs the question why the intraleucocytic organism was not detected by Cook *et al*. (2009), when they recorded *H. fitzsimonsi* in 35 tortoises of five species, *C. angulata*, *K. b. belliana*, *K. lobatsiana*, *K. natalensis* and *S. pardalis*, from the provinces of Gauteng, KZN, the North West, and the Western Cape. It also does not explain its absence from a further 34 tortoises, of five species, *C. angulata* (collected from the provinces of the NC and WC), *K. b. belliana* (from KZN), *K. lobatsiana* (from GP), *K. natalensis* (from KZN) and most importantly specimens of *S. pardalis* (collected from the provinces of GP and NC), all parasitized with *H. fitzsimonsi.*

Additionally, besides being much larger than both intraerythrocytic haemogregarines recorded previously from South Africa (Cook *et al*. 2009), namely *H. fitzsimonsi* and *H.parvula* (Fig. 1H,I), the intraleucocytic haemogregarine is also unlike them morphologically, especially in its cytoplasmic granularity. Despite apparently being confined to KZN, the intraleucocytic parasite is not encapsulated like *H. parvula* (Fig. 1H,I), which is found in tortoise erythrocytes in Mozambique (Dias 1953), and in KZN (Cook *et al*. 2009; present study). Future molecular work is certainly required to clarify this intraleucocytic parasite's relationship to both the above-mentioned intraerythrocytic haemogregarines.

Although the parasite could be grouped temporarily within the genus *Haemogregarina* based on Siddall's (1995) phylogenetic placement of all chelonian haemogregarines in the genus *Haemogregarina* (*sensu stricto*), we found no evidence that it undergoes intraerythrocytic or intraleucocytic (as in *H. pseudemydis*) division, or that it is leech-transmitted, both requirements for its inclusion in this genus (Siddall 1995). Although leeches may occur on land tortoises in Mkuze and Bonamanzi because of the subtropical environment, it is more likely that the intraleucocytic haemogregarine is tick-transmitted, as tortoise ticks have been found previously in several South African provinces (see Cook *et al*. 2009), and another land tortoise haemogregarine, *Hemolivia mauritanica* (Sergent & Sergent, 1904), has a tick vector (Široký *et al*. 2007). It may therefore align better with the genus *Hepatozoon*, species of which can develop in leucocytes and generally lack intraerythrocytic division (see Davies & Johnston 2000). Additional apicomplexans occurring in the leucocytes of reptiles include species parasitizing lizards within the haemococcidian genera

Schellackia Reichenow, 1919, *Lainsonia* Landau, 1973 and possibly, *Lankesterella* Labbé, 1899 (see Telford 2009). Members of all three genera lack merogony in circulating erythrocytes, but there are no current records of them in chelonians.

The characteristic features of the organism described here are its occurrence within leucocytes, its large size, especially its width (up to 10.6 μm), and its granularity. The gamont stages sometimes appear tightly recurved within a parasitophorous vacuole, suggesting they may be even longer/larger than current measurements suggest. Finally, the finding of an intraleucocytic haemogregarine infecting a South African terrestrial tortoise species is a novel observation and is here considered to be a *Hepatozoon* sp. Future research on this potentially unique species should aim to include molecular work to establish its proper taxonomic placement.

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