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Source: Journal of Wildlife Diseases, 37(1) : 12-19

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-37.1.12

Journal of Wildlife Diseases, 37(1), 2001, pp. 12–19

HEMOPROTOZOA OF FRESHWATER TURTLES IN QUEENSLAND

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ABSTRACT: Blood smears from 27 turtles (15 *Emydura signata*, nine *Elseya latisternum*, and three *Chelodina longicollis*) from southeastern Queensland (Australia) were examined for infections by hemoprotozoan parasites between January and June 1999. Infections were found in 26 (96%) of the turtles. Twenty five (93%) were infected with the adeleorin coccidian *Haemogregarina clelandi*, eight (30%) with the hemosporidian *Haemoproteus chelodinae*, 11 (41%) with the kinetoplastid flagellate *Trypanosoma chelodinae*, and eight (30%) with a novel *Trypanosoma* sp. Despite the high prevalence and intensity of infections, there was no evidence of clinical disease in any of the turtles.

Key words: Freshwater turtles, *Haemogregarina clelandi*, *Haemoproteus chelodinae*, hemoprotozoan parasite, survey, *Trypanosoma chelodinae*.

INTRODUCTION

Chelonians (turtles) belong to an ancient group dating back 200 million yr to the Jurassic era. Consequently, their parasites are of great importance from an evolutionary perspective due to their presumed ancient origins. Three species of freshwater turtles occur in southeastern Queensland (Cogger, 1996); the eastern snake-necked turtle (*Chelodina longicollis*), the saw-shelled turtle (*Elseya latisternum*), and the Brisbane River turtle (*Emydura signata*). Although many species of hemoprotozoa have been described worldwide, only three have been formally described from Australian chelonians; *Haemogregarina clelandi*, *Trypanosoma chelodinae*, and *Haemoproteus chelodinae* (cf. Mackerras, 1961). Little additional research has been conducted on the occurrence, identity, and host ranges of chelonian parasites in Australia. This paper records and describes four hemoprotozoan parasites present in freshwater turtles from southeastern Queensland.

MATERIALS AND METHODS

Freshwater turtles were collected from dams in the Brisbane River catchment at Pinjarra Hills in metropolitan Brisbane (Queensland, Australia; 27°32′S, 152°54′E) between January and June 1999. Turtles were trapped in wire mesh cages with inverted conical entrances, using fresh meat as bait. Traps were set at sunset and checked at sunrise. Individual animals were weighed, measured (carapace length), sexed, and marked with distinctive notches around the edge of the carapace. Turtles were housed in plastic aquaria with undergravel filters and raised basking platforms. Blood was collected from the femoral vein and thin blood smears were prepared immediately. The remaining blood was subjected to microhematocrit centrifugation (Clay-Adams, Parsippany, New Jersey, USA) at 2,000 × *G* and the buffy coat layer examined for trypanosomes. Blood smears were stained with Giemsa for 15 min and then examined at 400× magnification. Uninfected animals, and those with low-level infections, were released as soon as possible at the point of capture, while heavily-infected turtles were kept in captivity for up to 3 mo to monitor parasitemia. The size, shape and appearance of parasite developmental stages and infected and uninfected red blood cells were made using a calibrated eyepiece micrometer (Olympus, Tokyo). Measurements of trypomastigotes followed the recommendations of Hoare (1972) and included total length (L), width (W), length of free flagellum (FF), distance from anterior end to nucleus (NA), nucleus to kinetoplast (KN) and kinetoplast to posterior end (PK). A minimum of 20 developmental stages were measured for each parasite per slide and all values are expressed in micrometers (μm) as the arithmetic mean with the range given in parentheses. Statistical analyses were performed to determine the significance of any relationships between the prevalence of infection and the species, sex, length or weight of their hosts (Student's *t*-test, Microsoft Excel 5.0, Los Angeles, California, USA) and between parasitemia and hematocrit (regression analysis, Statistix®, Sydney, Australia).

TABLE 1. Morphometric characterisation (expressed in μm) of *Trypanosoma* spp. isolated from Australian freshwater turtles.

	L[a]	W[b]	PK[c]	KN[d]	NA[e]	FF[f]	Reference
T. chelodinae	34–38	5–8	5–9	8–16	11–17	15–19	Mackerras, 1961
T. chelodinae	39–43	5–9	5–8	10–12	18–21	3–6	Johnston and Cleland, 1910
Type 1 = *T. chelodinae*	55–70	6–7	3–7	12–20	15–23	17–28	Current study
Type 2 = *Trypanosoma* sp.	52–62	3–6	3–6	3–9	20–28	15–24	Current study
Type 3 = degenerative	52–83	3–8	0–7	10–34	20–28	14–29	Current study

[a] Length.
[b] Width.
[c] Distance from posterior end to kinetoplast.
[d] Distance from kinetoplast to nucleus.
[e] Distance from nucleus to anterior end.
[f] Length of free flagellum.

RESULTS

General

Twenty-six (96%) of the 27 turtles were infected with hemoparasites: 25 (93%) with an adeleorin coccidian identified as *H. clelandi*, eight of 27 (30%) with a hemosporidian identified as *H. chelodinae*, 11 (41%) with the kinetoplastid flagellate *T. chelodinae* and eight (30%) with a novel *Trypanosoma* sp. Monospecific infections (by one parasite only) were detected in 13 turtles (12 with *H. clelandi*, one with *Trypanosoma* sp.), dual infections were found in eight turtles (five with *H. clelandi* and *Trypanosoma* sp. and three with *H. clelandi* and *H. chelodinae*) and infections by all three parasite genera were found in five turtles. None of the turtles exhibited clinical signs of disease.

Trypanosomes

Trypanosomes were detected in six of 15 (40%) *E. signata*, four of nine (44%) *E. latisternum* and one of three (33%) *C. longicollis*. There were no significant differences in the prevalence of infection by trypanosomes according to host species, sex, length or weight ($P > 0.05$). The intensities of infection were generally low, with 1 to 10 trypanosomes detected in a whole blood smear. Infections were best detected by concentrating organisms in the buffy coat by microhematocrit centrifugation. In wet preparations, trypomastigotes were motile and swam in a jerky motion with rapid movements of the undulating membrane and free flagellum. Morphometric analyses of trypanosomes found during this study revealed three types of trypomastigotes differing in their sizes (especially width), kinetoplast position, and staining affinities (Table 1). Type 1 trypomastigotes were identified as those of *T. chelodinae* on the basis of their morphological characteristics. They were monomorphic in appearance, elongate and tapering, measuring 61.3 long × 6.7 wide (54.6–70.0 × 5.6–7.0), with a pointed posterior end. The granular cytoplasm stained dark pink with Giemsa while the prominent nucleus and kinetoplast stained purple (Fig. 1) The undulating membrane was well-developed. Type 2 trypomastigotes are considered to represent a novel *Trypanosoma* sp. on the basis of their morphological characteristics. These were monomorphic and slender, measuring 57.2 × 4.3 (51.8–62.3 × 2.8–5.6) with pointed posterior ends (Fig. 2) The distance between the kinetoplast and nucleus of these forms was consistently short (3–9). The granular cytoplasm stained blue to violet, with dark pink kinetoplasts and nuclei. Type 3 trypomastigotes exhibited poor morphological integrity and may be degenerative forms. They were long and tapering, measured 69.6 × 6.2 (52.5–82.6 × 3.5–8.4) and often assumed contorted shapes (Fig. 3). The free flagellum was well developed (14–29.4) and trypomasti-

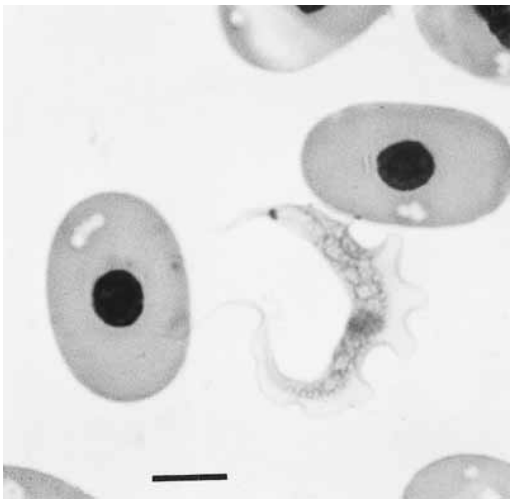

FIGURE 1. Trypomastigote of *Trypanosoma chelodinae* in *Emydura signata.* Giemsa stain. Bar = 8 μm.

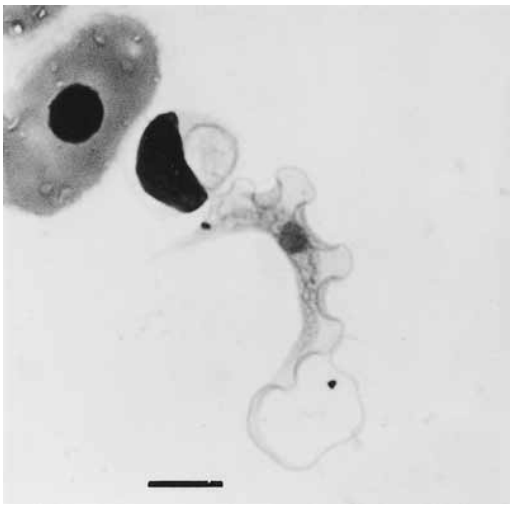

FIGURE 2. Thin-bodied trypomastigote of novel *Trypanosoma* sp. in *Emydura signata.* Giemsa stain. Bar = 8 μm.

gotes had a notably short PK distance (0–2.4). The cytoplasm, undulating membranes and nuclei stained light pink with Giemsa.

Hemosporidia

Infections by *H. chelodinae* were detected in five of nine (55%) *E. latisternum* and three of 15 (20%) *E. signata* but not in any of three *C. longicollis.* The parasites caused minimal host cell distortion or enlargement. No erythrocytic schizogony was observed. There was a significant difference in the prevalence of infection in the different turtle sexes ($P = 0.02$) with infections more prevalent in females (55%) than males (13%). No differences were observed in the prevalence of infection according to turtle species, length, and weight ($P > 0.05$). The parasitemia ranged from <1 to 50% but there were no significant relationships between parasitemia and host species, sex, age or hematocrit levels ($P > 0.05$).

All stages detected consisted of gamonts located intracellularly in host erythrocytes. Immature gamonts were circular to oval, with pink or blue granular cytoplasm depending on whether they were micro- (male) or macro- (female) gametocytes, respectively. They contained four or more vacuoles and dark pigment granules arranged in groups or scattered throughout the cytoplasm. Mature gamonts ranged from circular or oval forms 11 (8–13) in diameter (Fig. 4) to crescentic and halteridial forms measuring 13.8 × 7.5 (7–20 × 4–12) (Fig. 5). The parasites always occupied polar positions within host cells, except when displaced by hemogregarine

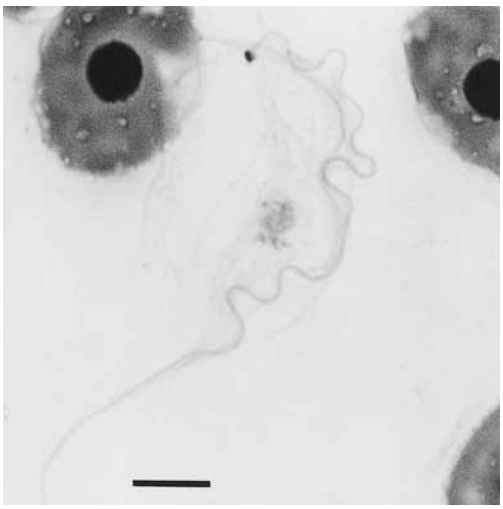

FIGURE 3. Degenerative trypomastigote in *Emydura signata.* Giemsa stain. Bar = 8 μm.

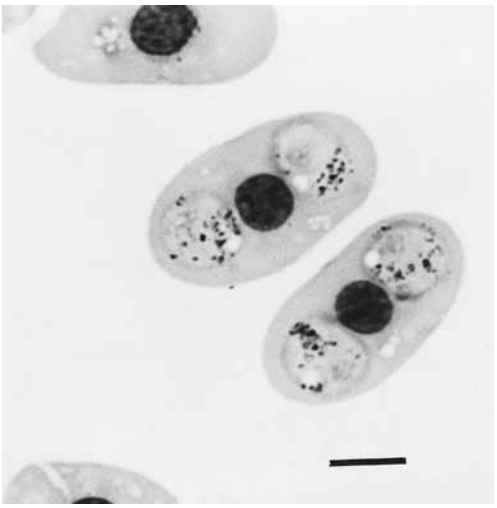

FIGURE 4. Mature gamonts of *Haemoproteus chelodinae* in *Elseya latisternum*. Giemsa stain. Bar = 8 μm.

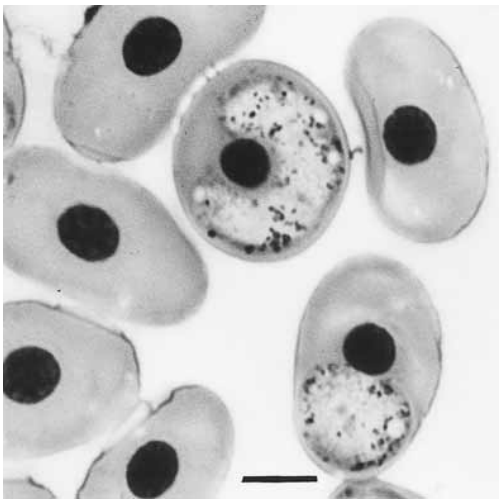

FIGURE 5. Halteridial forms of *Haemoproteus chelodinae* in *Elseya latisternum* often occupied up to 70% of the erythrocyte volume. Giemsa stain. Bar = 8 μm.

gamonts. Halteridial forms often occupied 50 to 70% of the cell volume. Macrogametocytes stained dark blue to violet and had coarse pigment granules grouped centrally, or at the periphery of the organism in clumps. These gamonts had between one and four vacuoles. Microgametocytes stained pink and had black pigmentation granules dispersed throughout cytoplasm, or occasionally at the periphery of the gamont. Yellow pigmentation granules were also observed in both micro- and macrogametocytes. At least one vacuole was present in each mature gamont.

Hemogregarines

Infections with *H. clelandi* were detected in 15 of 15 (100%) *E. signata,* seven of nine (77%) *E. latisternum* and all three (100%) *C. longicollis.* Parasitemia in infected hosts ranged between <1 to 12%. Minimal enlargement or distortion was caused to host erythrocytes by parasites, although host nuclei were usually displaced laterally or terminally. There were significant differences in the prevalence of infection between turtle species ($P = 0.005$), sexes ($P = 0.002$) and weights ($P = 0.01$) with infections being less prevalent in *E. latisternum,* in females (32%) than males (68%) and lighter turtles (36% in turtles <1 kg and 64% in turtles >1 kg). Parasitemia increased significantly with turtle length ($P = 0.04$) and weight ($P = 0.01$) but did not differ significantly with species or sex ($P > 0.05$). There was a significant positive correlation between parasitemia and hematocrit ($r = 0.63$, $P = 0.05$)

A variety of developmental stages were observed in blood films including schizonts, immature, mature and vermiform gamonts of *H. clelandi.* Intraerythrocytic schizonts were oval to bean-shaped, measured 15.5 × 7.1 (15.2–16.0 × 7.0–7.2) in size and contained 4 to 6 darkly-staining ovoid nuclei and lightly staining granular cytoplasm (Fig. 6). Immature gamonts were bean-shaped, circular or elongate in appearance (Fig. 7) and measured 9.2 × 4.6 (7.0–12.0 × 3.0–7.0) with nuclei measuring 4.1 × 4.5 (3.0–6.0 × 2.0–7.0). The gamonts had vacuolated pale pink cytoplasm with darkly-staining, distinct nuclei. No capsules were observed around these organisms. Infections of host cells with two mature gamonts were common. Maturing gamonts of *H. clelandi* were crescent-shaped, measured 18.7 × 7.3 (15–22 × 4–

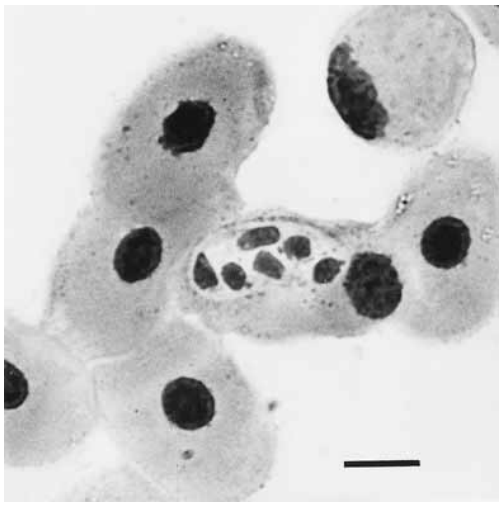

FIGURE 6. Intra-erythrocytic schizont of *Haemogregarina clelandi* in *E. signata.* Giemsa stain. Bar = 8 μm.

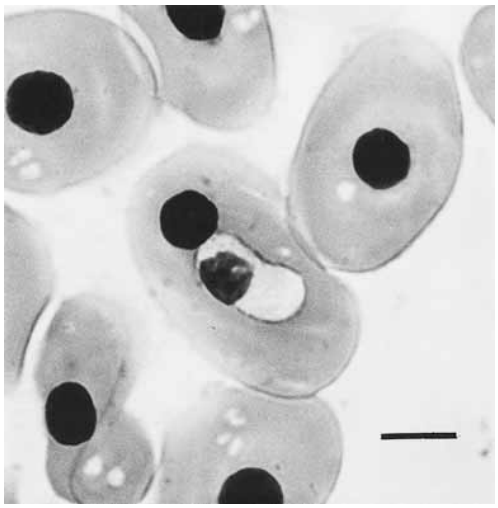

FIGURE 8. Maturing gamonts of *Haemogregarina clelandi* in *Emydura signata.* Giemsa stain. Bar = 8 μm.

13) in size, with darkly-staining nuclei measuring 4.3 × 5.5 (3–7 × 2–9), and were contained within vacuoles (Fig. 8). The gamont cytoplasm and nucleoplasm were highly granular. Parasites generally occupied lateral positions within host erythrocytes, causing lateral or terminal displacement of the host nucleus, but little enlargement or distortion of the host cell.

Mature gamonts of *H. clelandi* appeared as encapsulated vermiform organisms with reflexed "tails" (Fig. 9). In dorso-ventral view, the parasites were oval to crescent shaped, measured 12.3 × 5.7 (7.0–15.0 × 4.0–8.0) with central or polar nuclei measuring 5.3 × 4.6 (3.0–7.0 × 3.0–6.0). The granular cytoplasm stained dark blue with Giemsa. These gamonts caused displacement of the host cell nucleus and distor-

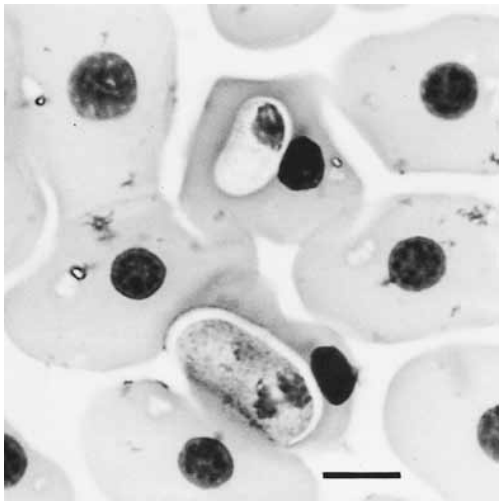

FIGURE 7. Immature gamont of *Haemogregarina clelandi* in *Emydura signata* (top). Mature macrogametocyte also present in lower portion of micrograph. Giemsa stain. Bar = 8 μm.

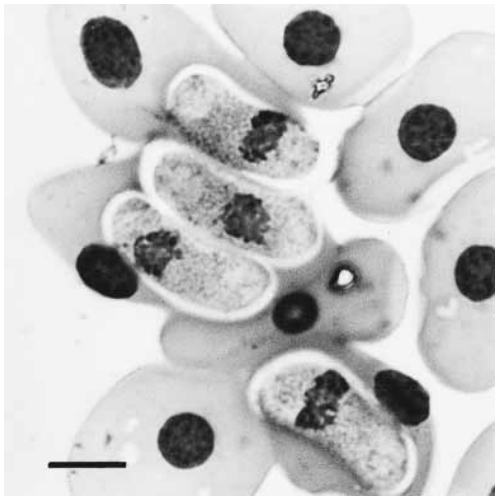

FIGURE 9. Mature gamonts of *Haemogregarina clelandi* in *Emydura signata.* Giemsa stain. Bar = 8 μm.

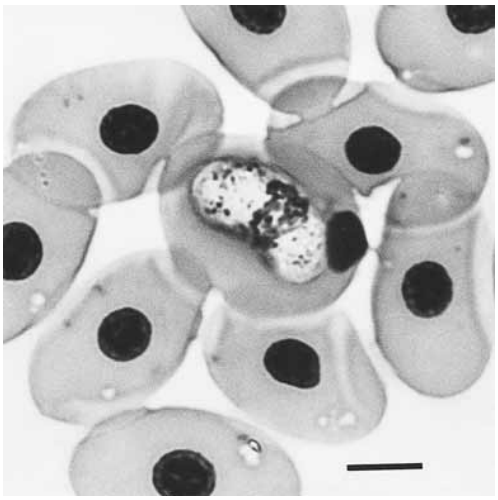

FIGURE 10. Unusual hemogregarine gamonts in erythrocytes of *Emydura signata.* Giemsa stain. Bar = 8 μm.

tion to the erythrocyte. In many cases, lysis of erythrocytes during the preparation of blood smears resulted in the presence of liberated, elongate gamonts and empty capsules in smears. Several unusual gamonts were also found in the erythrocytes of one *E. signata.* These morphotypes were also bean-shaped (12.8–17.6 × 2.4–6.4) and occupied lateral positions within erythrocytes. They stained distinctly from other mature gamonts, and had light pink or clear staining cytoplasm with a bright pink, stringy nucleus. No capsules were present. The presence of these organisms usually caused host cell enlargement or distortion, and lateral displacement of nuclei (Fig. 10). These forms have not previously been described from freshwater turtles.

DISCUSSION

The overall prevalence of blood protozoa in freshwater turtles in this study was 96%. Although the sample size was small (n = 27), the results suggest the frequent occurrence of parasites in freshwater turtles in this location in southeast Queensland. Infections were found in all three turtle species sampled. Few differences in the prevalence of infection were found between different turtle species, sexes or age groups (as determined by length or weight). This suggests all turtle species were susceptible to infection and there was no apparent age-related resistance to infection.

Only one *Trypanosoma* sp. has been previously described from freshwater turtles in Australia, namely *T. chelodinae* from *C. longicollis, Emydura kreftii, E. macquarii, E. latisternum* and *E. dentata* (cf. Mackerras, 1961). Many others have been described from chelonian hosts elsewhere in the world (Woo, 1969; Molyneux, 1977). Although some aquatic reptiles have trypanosomes transmitted by arthropod vectors, e.g., *T. grayi* of crocodiles transmitted by tsetse flies (Telford, 1991), the vectors for turtle trypanosomes are presumed to be placobdellid leeches (Stone, 1976). These are commonly found on freshwater turtles and are known to transmit a wide range of hematozoa in addition to trypanosomes (Siddall and Burreson, 1996). The prevalence of trypanosome infection in Australian turtles was 41% which is much higher than that reported previously for other reptilian and amphibian hosts (Breinl, 1913; Woo, 1969). More infections may have been detected in this study due to more stringent examination techniques, namely microhematocrit centrifugation. Indeed, the intensities of infection were often low and many infections were not evident on examination of thin smears.

Three types of trypomastigotes were detected in the turtles. One type was identified as the species *T. chelodinae* as described previously (Johnston and Cleland, 1910; Mackerras, 1961) except that the trypomastigotes apparently were longer. However, previous measurements did not include the length of the free flagellum as recommended by Hoare (1972). When length measurements were adjusted to include the free flagellum, the morphometric characteristics of *T. chelodinae* were remarkably consistent not only between dif-

ferent turtle species, but also between the different studies.

The second type of trypomastigote appears to represent a novel species which differed significantly in width ($P < 0.001$), nucleus to anterior ($P < 0.01$) and kinetoplast to nucleus ($P < 0.0001$) distances. Other characteristics, such as total length, distance from posterior to kinetoplast, and length of the free flagellum were similar to *T. chelodinae*. Trypomastigotes in the bloodstream of vertebrate hosts divide asexually by longitudinal binary fission, and all major organelles (including the kinetoplast and nucleus) of the progeny maintain constant anatomical positions. It is therefore unlikely that the second type of trypomastigotes are merely developmental variations of *T. chelodinae* but rather represent a novel species in freshwater turtles.

In contrast, the third type of trypomastigote was considered to represent degenerative forms. The trypomastigote body exhibited very weak staining affinities, the nucleus was hard to discern and appeared hypertrophied and the flagellum was poorly stained although the kinetoplast was prominent and located close to the posterior pole. Dividing stages are usually condensed and contain darkly-staining organelles whereas these stages appeared turgid and close to lysis. They were not as prevalent in smears as the two distinct *Trypanosoma* spp. but they occurred with sufficient regularity to be recorded here. Further studies are required to determine the survival and staining characteristics of trypomastigotes under a range of conditions to differentiate between normal biological variation and preparative artifacts.

Hemogregarines were the most prevalent parasite of freshwater turtles in this study, with an overall prevalence of 93%. *Emydura signata* in particular appears to be highly susceptible to infection with 100% of individuals infected. The intensities of infection were highly variable with the percentage parasitemia ranging from <1 to 12%. A significant correlation was detected between % parasitemia and hematocrit with higher hematocrits found in heavily infected turtles suggesting infections do not cause red cell depletion. Indeed, gamonts do not lyse host cells but remain dormant within the cells until they are taken up by an invertebrate vector. It is not known whether hemogregarines affect erythrocyte longevity or whether they stimulate host erythropoiesis thereby contributing to higher hematocrits. The situation may be compounded by other parameters as the intensity of infection appeared to differ according to season and host age. Infections seemed to be heavier during the winter which is when turtles hibernate, and they were heavier in older turtles suggesting they accumulate with age. Mackerras (1961) previously observed that turtles tend to develop an ongoing tolerance to these parasites rather than developing immune responses to them.

A variety of hemogregarine developmental stages were seen in blood films from all three turtle species; schizonts, immature gamonts and mature vermiform gamonts. The finding of schizonts in the circulating blood confirms the identity of these parasites as *Haemogregarina* sp. rather than *Hepatozoon* sp. The mature gamonts were similar in size, shape and appearance to those of *H. clelandi* described by Mackerras (1961), and *H. balli* described by Paterson and Desser (1978). However, as noted by Mackerras (1961) the identification of *Haemogregarina* spp. solely on the basis of gamont morphology and host occurrence is problematic. It is now generally accepted that other characters, such as developmental cycle and invertebrate vector should be included in differential diagnostic studies. Regrettably, the vector of *H. clelandi* is not yet known and little information is available on stages other than gamonts.

The hemosporidian parasite detected in the turtles was identified as a *Haemoproteus* sp. on the basis of the presence of hemozoin pigment and the absence of intraerythrocytic schizogony. The taxonomy

of chelonian hemoproteids remains fluid, as most species of *Haemoproteus* have been classified purely on the basis of host occurrence without reference to developmental cycle or invertebrate vector. Nonetheless, the morphology of some of the hemoproteid stages corresponded to that of *H.* (=*Haemocystidium*) *chelodinae* (Johnston and Cleland, 1910). On occasions, gametocytes appeared to wrap around the host cell nucleus, giving them a distinct crescentic or halteridial shape. Halteridial gametocytes of *Hp. chelodinae* were not noted by Mackerras (1961) although an earlier study of turtle hemoparasites by Shortt (1922) found that halteridia were often the predominating form in blood cells, making them indistinguishable from avian hemoproteids. It is probable, therefore, that halteridia found in this study represent transient developmental stages of the hemoproteids. The other morphotypes encountered in this study have not been previously described, and further studies are required to determine their relationships to described forms.

The overall prevalence of hemoprotozoan parasites in turtles (96%) combined with consistently high parasitemia and an apparent lack of pathology to hosts during this study suggests that these particular host-parasite associations have evolved over a long period of time. Electron microscopy and molecular analyses should help to clarify the phylogenetic relationships of these hemoparasitic assemblages to each other, and to members of other groups.

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Received for publication 7 December 1999.