Morphologic and Morphometric Analysis of *Hepatozoon* spp. (Apicomplexa, Hepatozoidae) of Snakes

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Hepatozoon *species are the most abundant hemoparasites of snakes. Its identification has been based mainly on the morphologic characterization of the gamonts in the peripheral blood of the vertebrate host and also of the cysts found in the internal organs of the vertebrate and invertebrate hosts. Using a computerized image analysis system, we studied five species of* Hepatozoon *from recently captured snakes in Botucatu, State of São Paulo, Brazil, to evaluate the importance of the morphology and morphometry of the gamonts for the characterization of* Hepatozoon *species and to analyze the morphologic changes induced in the erythrocytes by the parasite. The studied species were* H. terzii *of* Boa constrictor amarali, Hepatozoon *sp. of* Crotalus durissus terrificus, H. philodryasi *of* Philodryas patagoniensis, *and* H. migonei *and* H. cyclagrasi *of* Hydrodynastes gigas. *We observed three different groups, one of them including the species* H. terzii, H. philodryasi *and* Hepatozoon *sp. of* C. durissus terrificus*; and the other two consisting of* H. migonei *and* H. cyclagrasi. *Degree of alterations in the erythrocytes was variable and it may be useful for characterization of* Hepatozoon *species.*

Key words: *Hepatozoon* - parasites - snakes - morphometry - morphology - erythrocytes

Hepatozoon species are the most abundant hemoparasites of snakes (Wozniak et al. 1994). Many *Hepatozoon* species have been described among Brazilian snakes: *H. juxtanuclearis* (Carini 1947, Pessoa 1967), *H. fusifex* (Ball et al. 1969) and *H. terzii* (Sambon 1909) parasites of *Boa constrictor*, *H. romani* and *H. capsulata* (Phisalix 1931) parasites of *Crotalus durissus terrificus*, *H. philodryasi* (Carini 1910) parasite of *Philodryas patagoniensis*, *H. migonei* (Arantes 1934, Schouten 1934, Pessoa et al. 1970) and *H. cyclagrasi* (Pessoa et al. 1970) parasites of *Hydrodynastes gigas*.

Identification of *Hepatozoon* species has been based mainly on the morphologic characterization of the gamonts in the peripheral blood of the vertebrate host and also of the cysts found in the internal organs of the vertebrate and invertebrate hosts. However, these stages are very similar for the different species of *Hepatozoon*, and so the identification based only on these characteristics may be incorrect (Pessoa & De Biasi 1973, Pessoa et al. 1973).

The main reports of Brazilian snake *Hepatozoon* give information on the morphology of the gamonts (shape, stain, and capsule presence) and also provide morphometric data usually consisting of the description of the length and width of the gamonts and cysts. In recent works, using computerized image analysis systems, measurements of the parasite and nucleus areas have been also reported (Silva et al. 1999). Morphometric analysis allows a more detailed evaluation of cellular morphology, including linear and area measurements.

Morphological descriptions of most *Hepatozoon* species of Brazilian snakes are of fundamental importance to contribute to the knowledge of this group of parasites. A complete morphological description of a *Hepatozoon* species should include morphological and morphometric features of all stages of the parasite, including the sporogonic stages in the arthropod vector.

The objective of the present investigation was to study species of *Hepatozoon* in recently captured snakes from Botucatu, State São Paulo, Brazil, to evaluate the importance of morphology and morphometry for the characterization of the species, and to analyze the morphologic alterations induced in the erythrocytes by the presence of the parasite.

MATERIALS AND METHODS

Snakes and blood collection - Parasites of four snake species from Botucatu, São Paulo, Brazil, were studied. The animals were donated to the Center for the Study of Venoms and Venomous Animals of São Paulo State University and presented *Hepatozoon* on the occasion of the first blood examination. Blood was collected from all snakes by ventral tail venipuncture and blood smears were prepared immediately upon collection. Smears were air dried, fixed with absolute methanol for 3 min and stained with 10% Giemsa for 30 min. The slides were microscopically surveyed for hemoparasites by light microscopy at 250x magnification.

The studied hosts and parasites were:

Boa constrictor amarali (Boidae) - The following species have been described for this snake: *H. juxtanuclearis*, *H. fusifex*, and *H. terzii* (Sambon 1909, Carini 1947, Pessoa 1967, Ball et al. 1969). The species *H. juxtanuclearis* (Carini

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Received 16 January 2002
Accepted 6 September 2002

1947) and *H. fusifex* (Ball et al. 1969) are morphologically different from the species studied in the present investigation that is *H. terzii* (Sambon 1909). The gamonts of *H. terzii* are club-shaped organisms with the anterior end broad and rounded, the posterior extremity tapering slightly to a blunt point and folded back hookwise for a length of about 3 µm. They are enclosed within capsules measuring 14 µm in length and 4 µm in breadth. The nucleus measure 3 µm in length by 4 µm in breadth, and is usually placed in the posterior half of the body. A variable number of deeply-staining granules are commonly present in the cytoplasm on either side of the nucleus (Sambon 1909).

Philodryas patagoniensis (Colubridae) - The only species described for this snake is *H. philodryasi* (Carini 1910). This species measures 11-13 µm in length and 2-3.5 µm in width. The parasitized erythrocytes are not changed by the parasites and the nucleus maintains its normal position (Carini 1910).

Hydrodynastes gigas (Colubridae) - Two *Hepatozoon* species have been described in the literature: a small one, *H. migonei* (Arantes 1934, Schouten 1934, Pessoa et al. 1970), and a large one, *H. cyclagrasi* (Pessoa et al. 1970). *H. migonei* is a parasite which presents 12-14 µm in length and 3-3.5 µm in breadth (Arantes 1934, Schouten 1934, Pessoa et al. 1970). *H. cyclagrasi* is reported as an abnormal form, with large cytoplasm, with "swollen" and "spongy" aspect, and it induces enlargement of the erythrocyte and displacement of nucleus for one of the cell poles. The parasite can measure up to 35-40 µm length (Pessoa et al. 1970).

Crotalus durissus terrificus (Viperidae) - The following species have been described in the literature: *H. romani* and *H. capsulata* (Phisalix 1931). *H. romani* measures 17-18 µm in length and 8.4 µm in width. The nucleus measures 2-3 µm and was not condensed. *H. capsulata* measures 16.8 µm in length and 6.3 µm in width. The nucleus measures 4.2 µm and was condensed (Phisalix 1931). The *Hepatozoon* studied in present investigation was not similar to any of the described species and therefore we considered it as *Hepatozoon* sp.

Morphologic and morphometric analyses - For each *Hepatozoon* species we analyzed the shape of the gamont, the presence of pigment in the cytoplasm, and the format and position of its nucleus. Morphometric analysis was performed using the KS-300 computerized image analysis system (Zeiss) at 1000x magnification. The analyzed variables were: area, length and width of the parasite, and area, length and width of the nucleus of the parasite. A total of 100 parasites were analyzed for each infected snake. We also analyzed 100 normal erythrocytes and 100 infected erythrocytes in terms of the following variables: area, length and width of the erythrocyte, and area, length and width of the nucleus of the erythrocyte.

Statistical analysis - Data obtained for the parasites were compared by analysis of variance, by the Student-Newman-Keuls test and by multivariate analysis. Data for infected and non-infected erythrocytes were compared by the t test. All statistical tests were performed using the Sigma Stat 2.0 and MVSP 3.1 software, with the level of significance set at 5%.

RESULTS

Morphologic analysis of the parasites

Boa constrictor amarali - *H. terzii* presented an elongated and slender shape, with a cellular area of 35.3 ± 4.1 µm^2; the length was 14.6 ± 0.4 µm and the width 2.6 ± 0.4 µm. The cytoplasm was slightly stained with no granulations. The nucleus was homogeneous, dense and parallel to the larger axis of the parasite. In 57.3% of the parasites the nucleus was slightly displaced towards one of the extremities and in 42.7% it was in a central position. The area of the nucleus was of 9.2 ± 1.6 µm^2, the length was 4.6 ± 0.9 µm and the width 2 ± 0.3 µm (Fig. 1A). Some erythrocytes were infected with more than one parasite.

Crotalus durissus terrificus - The *Hepatozoon* sp. studied presented an elongated and slender shape. Its cellular area was of 33.2 ± 4.1 µm^2, the length was 14.7 ± 0.6 µm and the width 2.4 ± 0.4 µm. The cytoplasm was lightly stained and homogeneous. The nucleus was homogeneous, dense, and parallel to the longer axis of the parasite. In 51.5% of the parasites the nucleus was lightly displaced towards one of the extremities and in 48.5% it was in a central position. Its area was 8.5 ± 1.7 µm^2, its length 5.1 ± 0.8 µm length and its width 1.7 ± 0.4 µm (Fig. 1B).

Philodryas patagoniensis - *H. philodryasi* presented an elongated and slender shape, with a cellular area of 37.5 ± 7.2 µm^2, a length of· 14.3 ± 1.1 µm length and a width of 2.8 ± 0.5 µm. Its cytoplasm was homogeneous and lightly stained. The nucleus was homogeneous, dense and parallel to the larger axis of the parasite. In 93.7% of the parasites the nucleus was lightly displaced toward one of the extremities and in 6.3% it was in a central position. Its area was of 9.6 ± 1.8 µm^2, its length 5.2 ± 0.8 µm and its width 2.0 ± 0.4 µm (Fig.1C). More than one gamont infecting the same erythrocyte was usually found in this host.

Hydrodynastes gigas - This snake presented two different parasites: (1) *H. cyclagrasi*, a large parasite almost occupying the entire cytoplasm of the erythrocyte, presented an elongated and wider shape. Its cellular area was 115.2 ± 11.8 µm^2, its length 21.7 ± 1.4 µm and its width 6.4 ± 0.8 µm. The parasite cytoplasm presented granulations and deep staining. The nucleus was homogeneous and arranged perpendicularly to the longer axis of the parasite. In 76.1% of the parasites the nucleus was slightly displaced towards one of the extremities and in 23.9% it was in a central position. Its area was 13.9 ± 2.6 µm^2, its length 5.1 ± 0.5 µm and its width 3.3 ± 0.6 µm (Fig. 1D). A "hook" was usually observed at one of the extremities of the parasite; (2) *H. migonei*, the smaller form was less elongated and more oval in shape. Its cellular area was 48.6 ± 5.9 µm^2, its length 11.8 ± 1.1 µm and its width 5 ± 0.6 mm. Its cytoplasm was homogeneous and lightly stained and contained a round and large nucleus in relation to the size of the parasite. In 52.5% of the parasites the nucleus was slightly displaced towards one of the extremities and in 47.5% it was in a central position. The area of the nucleus was 13.3 ± 2.7 µm^2, the length was 4.5 ± 0.6 µm and the width 3.5 ± 0.6 µm (Fig. 1E).

Comparative analysis of the various parasites - Analysis of parasite area, length and width showed that the parasites of the various species had different dimensions ($p < 0.05$), except for the length of *Hepatozoon* sp. of *C. durissus terrificus* and *H. terzii* ($p > 0.05$) (Table I). The various parasites also differed in terms of nucleus area and width ($p < 0.05$), except for *H. philodryasi* and *H. terzii* ($p > 0.05$) (Table I). The parasites showed two different patterns in terms of nuclear length: one shared by *H. philodryasi*, *Hepatozoon* sp. of *C. durissus terrificus* and *H. cyclagrasi*, and the other shared by the species *H. terzii* and *H. migonei* (Table I). Multivariate analysis showed that the studied parasites can be divided into three different groups: one of them includes the species *H. terzii, H. philodryasi* and *Hepatozoon* sp. of *C. durissus terrificus*; and the other two consist of *H. migonei* and *H. cyclagrasi* (Fig. 2).

Comparative analysis of infected and non-infected erythrocytes - Comparative analysis of infected and non-infected erythrocytes (t test) showed that the values obtained for 73.3% of the analyzed variables were significantly different ($p < 0.05$) (Table II). We observed that linear dimensions (nuclear or erythrocyte length and width) were different in 85% of the cases, whereas area dimensions were different in only 50.0% of the cases (Table II).

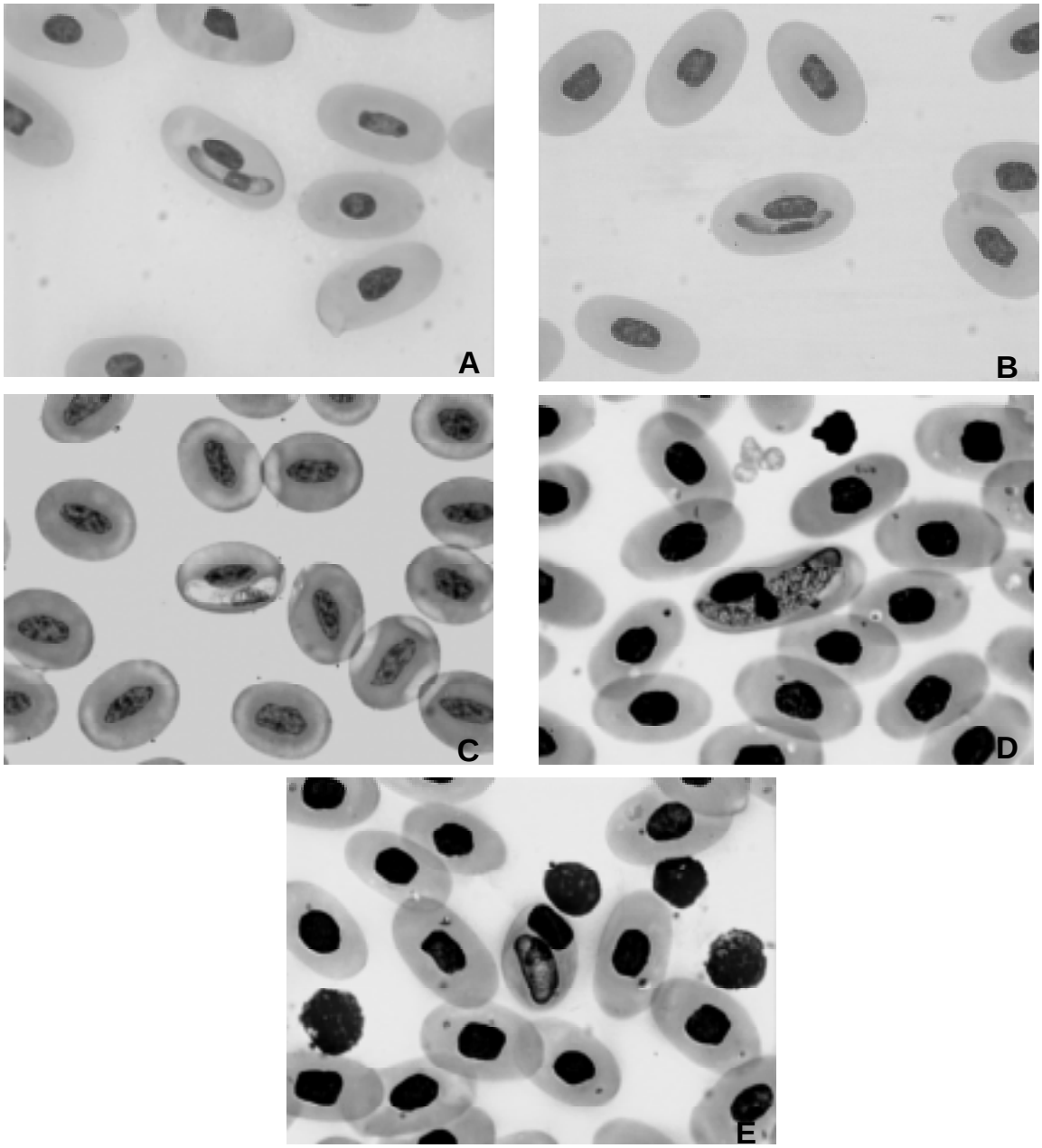

Fig. 1: gamont of *Hepatozoon* spp. in a blood smear. A: *H. terzii* from *Boa constrictor amarali*; B: *Hepatozoon* sp. from *Crotalus durissus terrificus*; C: *H. philodryasi* from *Philodryas patagoniensis*; D: *H. cyclagrasi* from *Hydrodynastes gigas*; E: *H. migonei* from *H. gigas*. Magnification, 1000x.

TABLE I

Comparative analysis of the *Hepatozoon* species studied (n = 100 for each species)

	Parasite			Nucleus		
Parasites	Area (μm^2)	Length (µm)	Width (µm)	Area (μm^2)	Length (µm)	Width (µm)
H. terzii	35.3 ± 4.2 a	14.6 ± 0.4 a	2.6 ± 0.4 a	9.2 ± 1.6 a	4.6 ± 0.9 a	2.0 ± 0.3 a
H. philodryasi	37.5 ± 7.2 b	14.3 ± 1.1 b	2.8 ± 0.5 b	9.6 ± 1.8 a	5.2 ± 0.8 b	2.0 ± 0.4 a
Hepatozoon sp.	33.2 ± 4.1 c	14.7 ± 0.6 a	2.4 ± 0.4 c	8.5 ± 1.7 b	5.1 ± 0.8 b	1.7 ± 0.4 b
H. migonei	48.6 ± 5.9 d	11.8 ± 1.1 c	4.9 ± 0.6 d	13.3 ± 2.7 c	4.5 ± 0.6 a	3.5 ± 0.6 c
H. cyclagrasi	115.2 ± 11.8 e	21.7 ± 1.4 d	6.4 ± 0.8 e	13.9 ± 2.6 d	5.1 ± 0.5 b	3.3 ± 0.6 d

The values presented in the Table represent the mean and the standard deviation for each variable; same letters in the columns represent $p > 0.05$; different letters in the columns represent $p < 0.05$.

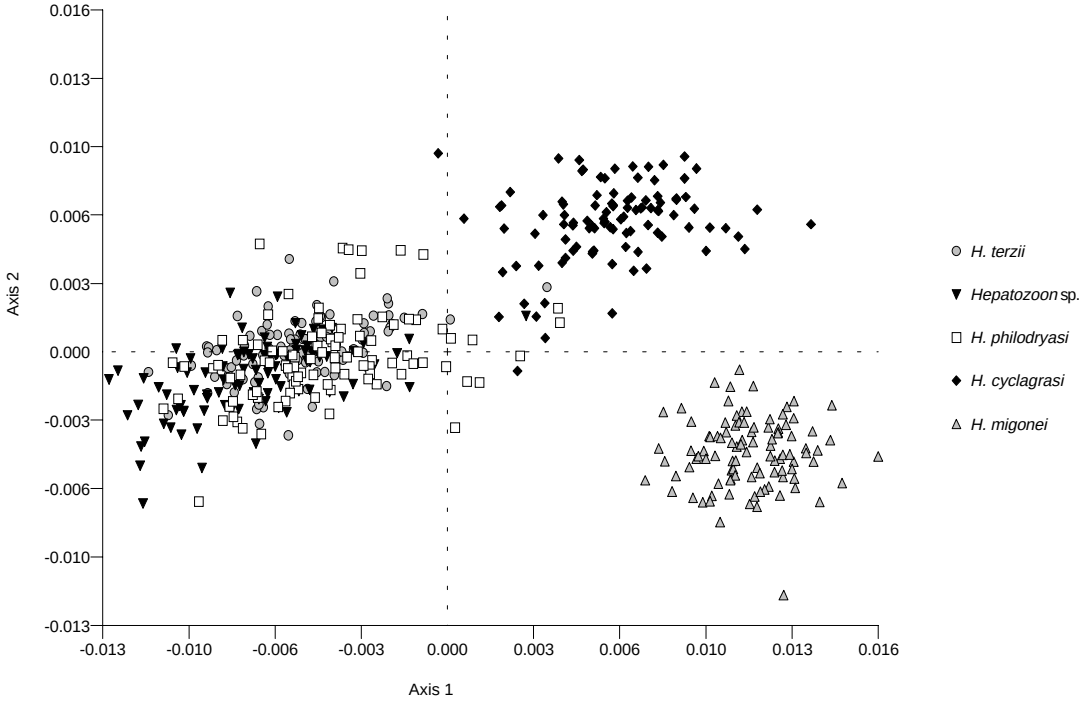

Fig. 2: multivariate analysis of the main components for comparison of the species of *Hepatozoon* studied. The values presented on the ordinate and abscissa axes represent the largest amount of variation in the data set.

Multivariate analysis, which was applied only to the linear variables, showed that *H. cyclagrasi* induced strong changes in erythrocyte morphology (Fig. 3). The changes in erythrocyte morphology induced by the species *H. terzii*, *Hepatozoon* sp. of *C. durissus terrificus* and by *H. migonei* were less obvious than the changes induced by *H. cyclagrasi* (Figs 4, 5, 6). The changes induced by *H. philodryasi* were not evident (Fig. 7).

H. terzii, *Hepatozoon* sp. of *C. durissus terrificus* and *H. philodryasi* induced light flattening of the erythrocyte nucleus, which, however, was not dislocated to the cell periphery of. In contrast, *H. cyclagrasi* dislocated the erythrocyte nucleus to one of the poles in 55.5% of cases and laterally in 44.5%, and also induced a marked deformation of the erythrocyte. *H. migonei* caused displacement of the erythrocyte nucleus to the poles in 63.1% of cases and laterally in 36.9%. In spite of this displacement, the changes in the erythrocyte were less evident.

DISCUSSION

The objective of the present study was to evaluate the importance of morphology and morphometry for the characterization of *Hepatozoon* species that parasitize snakes. The dimensions and morphology of the five *Hepatozoon* species analyzed are in agreement with the description reported in the literature for those species. We evaluated linear and area data of the gamonts and submitted them to multivariate analysis, which permitted us to divide the species into three different populations (Fig. 2).

In most reports on *Hepatozoon* parasitizing snakes, new species were described on the basis of the length and width of the gamonts and schizonts and also of oocysts, sporocysts and sporozoites (Pessoa & Biasi 1973). Also, in a recent review article it was reported that many *Hepatozoon* species were created by simply considering a new infected host (Smith 1996).

The literature reports that exist immature and mature gamonts and that these developmental stages could be interpreted as different species (Smith 1996). Snakes with actively merogony may present gamonts of different sizes, with more slender forms representing an immature precursor to the larger gamonts (Smith et al. 1994). In the present paper, the specimens of the five studied species were homogeneous and the differentiation between mature and immature forms was not observed.

Some reports have demonstrated that the same *Hepatozoon* species can infect different hosts (Hull & Camin 1960, Pessoa et al. 1971). Besides, the gamonts of a same species of *Hepatozoon* can present slight morphologic changes depending on the host. Pessoa et al. (1974) demonstrated that it was possible to transfer *H. tupinambis* of the lizard *Tupinambis teguixin* to the snake *C. durissus terrificus*. The transfer induced changes in the parasite so that in the lizard, *Hepatozoon* species produced eryth-

TABLE II

Comparative analysis of normal and parasitized erythrocytes of different snakes species

	Cell			Nucleus		
Parasites	Area (μm^2)	Length (µm)	Width (µm)	Area (μm^2)	Length (µm)	Width (µm)
Boa constrictor amarali						
Normal	161.3 ± 16.1	19.1 ± 1.1	10.2 ± 0.7	19.3 ± 3.0	6.1 ± 0.9	3.7 ± 0.4
Parasitized	159.8 ± 18.2	19.7 ± 1.2 *a*	9.7 ± 0.7 *a*	20.0 ± 2.8	7.3 ± 0.7 *a*	3.2 ± 0.5 *a*
Crotalus durissus terrificus						
Normal	162.4 ± 21.2	18.9 ± 1.3	10.6 ± 0.9	24.2 ± 3.9	6.7 ± 0.9	4.3 ± 0.5
Parasitized	172.9 ± 27.0 *a*	20.3 ± 1.6 *a*	10.4 ± 1.1	24.7 ± 4.8	7.6 ± 0.8 *a*	4.0 ± 0.6 *a*
Philodryas patagoniensis						
Normal	155.6 ± 24.4	17.5 ± 2.3	11.2 ± 1.0	23.4 ± 3.6	7.5 ± 1.0	4.0 ± 0.7
Parasitized	167.6 ± 19.0 *a*	18.9 ± 2.4 *a*	11.2 ± 1.1	22.6 ± 5.8	7.7 ± 0.8	3.7 ± 0.8 *a*
Hydrodynastes gigas – larger parasites						
Normal	192.4 ± 14.1	20.7 ± 1.1	11.6 ± 0.7	33.2 ± 3.9	7.5 ± 0.7	5.4 ± 0.5
Parasitized	187.9 ± 21.9	20.1 ± 1.5 *a*	11.9 ± 1.3 *a*	29.7 ± 2.8 *a*	7.3 ± 0.6 *a*	5.1 ± 0.5 *a*
Hydrodynastes gigas – smaller parasites						
Normal	192.4 ± 14.0	20.7 ± 1.0	11.6 ± 0.7	33.2 ± 3.3	7.5 ± 0.6	5.4 ± 0.4
Parasitized	252.7 ± 27.7 *a*	25.4 ± 1.5 *a*	12.4 ± 1.6 *a*	26.8 ± 4.4 *a*	8.5 ± 0.6 *a*	3.9 ± 0.5 *a*

a: statistically significant differences in relation to normal erythrocytes.

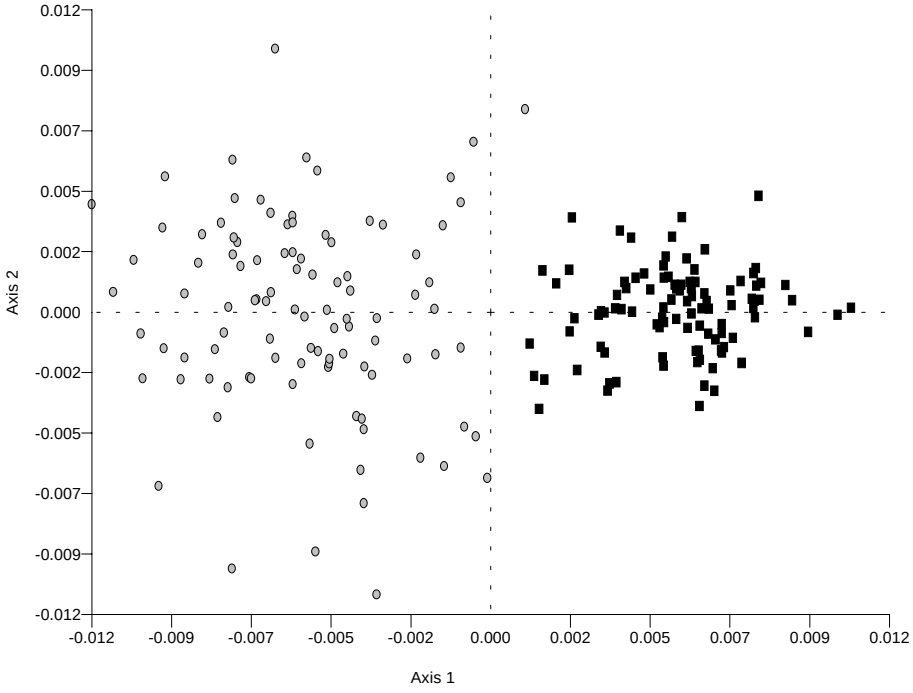

Fig. 3: multivariate analysis of the main components for comparison of normal erythrocytes (■) (n = 100) and erythrocytes parasitized (●) (n = 100) by *Hepatozoon cyclagrasi* of *Hydrodynastes gigas*. The values presented on the ordinate and abscissa axes represent the largest amount of variation in the data set.

rocyte deformations but this characteristic was gradually lost in the experimental infections.

The possibility that all of the *Hepatozoon* species described as parasites of terrestrial snakes, which are transmitted by mosquitoes, constitute a single species whose blood forms vary with the vertebrate host has also been discussed in the literature (Ball 1970, Pessoa et al. 1971).

The above considerations show that a controversy exists on this subject. Some investigators believe that several *Hepatozoon* species exist while others discuss the possibility that it is a single species presenting different morphologic patterns depending on the infected host (Smith 1996).

Our results show three different populations, support-

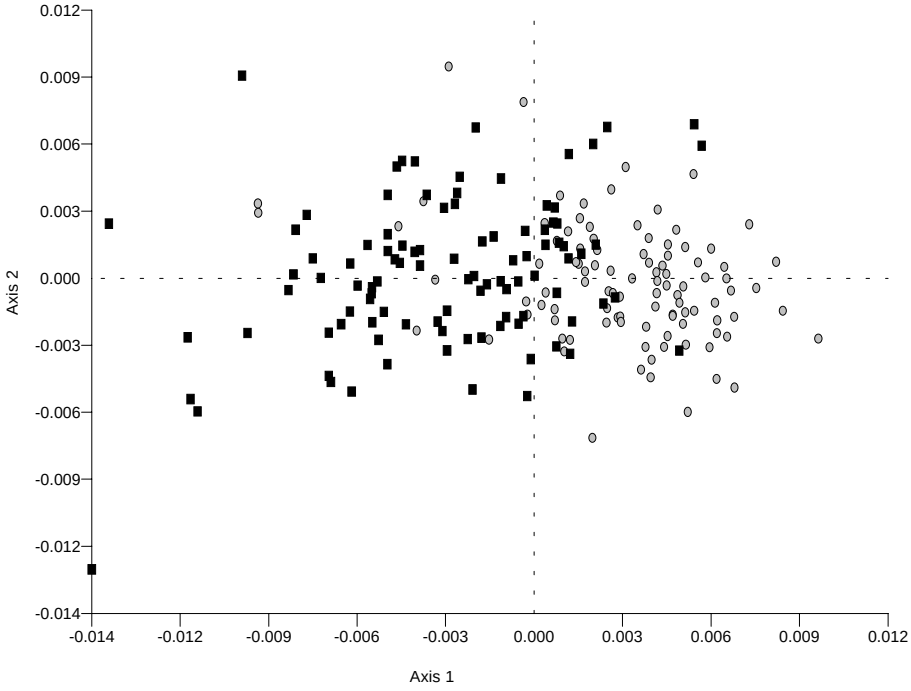

Fig. 4: multivariate analysis of the main components for comparison of normal erythrocytes (■) (n = 100) and erythrocytes parasitized (●) (n = 100) by *Hepatozoon terzii* of *Boa constrictor amarali*. The values presented on the ordinate and abscissa axes represent the largest amount of variation in the data set.

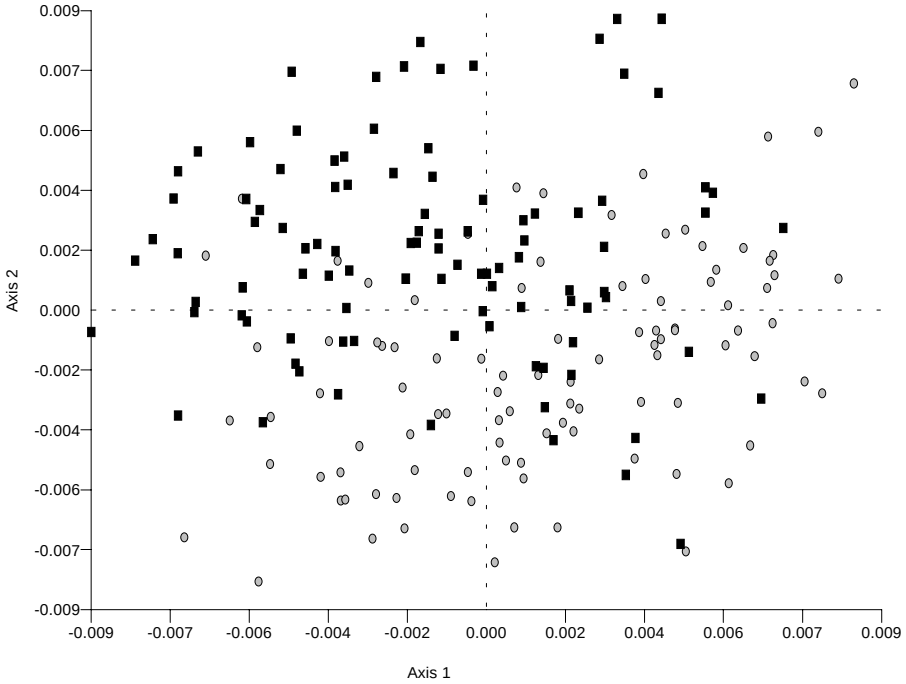

Fig. 5: multivariate analysis of the main components for comparison of normal erythrocytes (■) (n = 100) and erythrocytes parasitized (●) (n = 100) by *Hepatozoon* sp. of *Crotalus durissus terrificus*. The values presented on the ordinate and abscissa axes represent the largest amount of variation in the data set.

ing the hypothesis of the existence of several species of *Hepatozoon* that can infect different species of snakes. The parasites found in *H. gigas* are significantly different among themselves and the two are different from the other studied parasites.

By comparing the present results with those reported in the literature about the *Hepatozoon* parasites of the same snakes studied by us, we observe that disagreement exists about the determination of the species. Our results show that the gamonts of the species *H. terzii, H. philodryasi* and the *Hepatozoon* sp. of *C. durissus terrificus* do not differ morphologically, as shown in other reports describing these species (Sambon 1909, Carini 1910, Phisalix 1931).

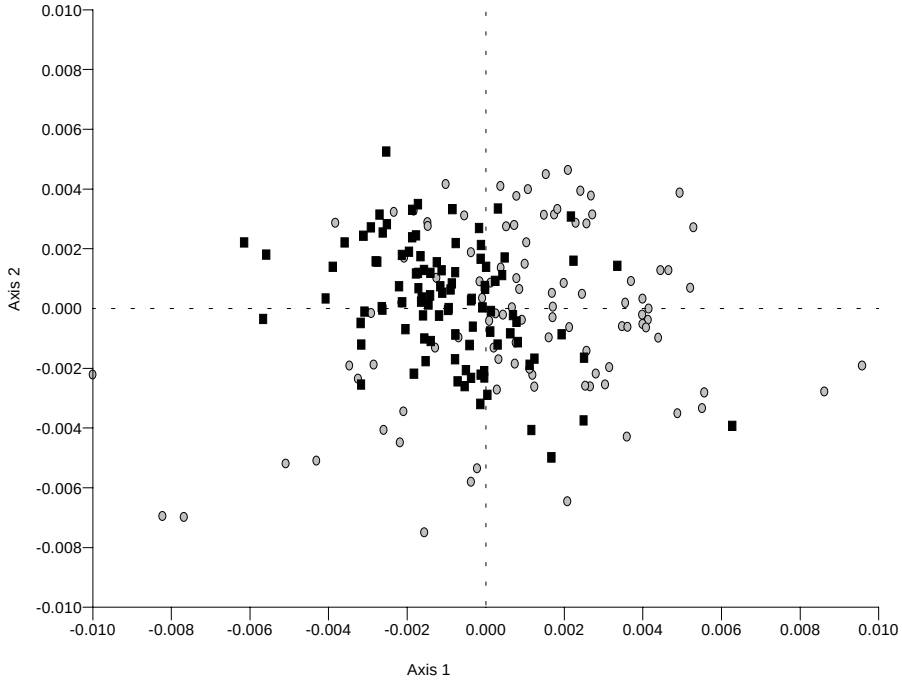

Fig. 6: multivariate analysis of the main components for comparison of normal erythrocytes (■) (n = 100) and erythrocytes parasitized (●) (n = 100) by *Hepatozoon migonei* of *Hydrodynastes gigas*. The values presented on the ordinate and abscissa axes represent the largest amount of variation in the data set.

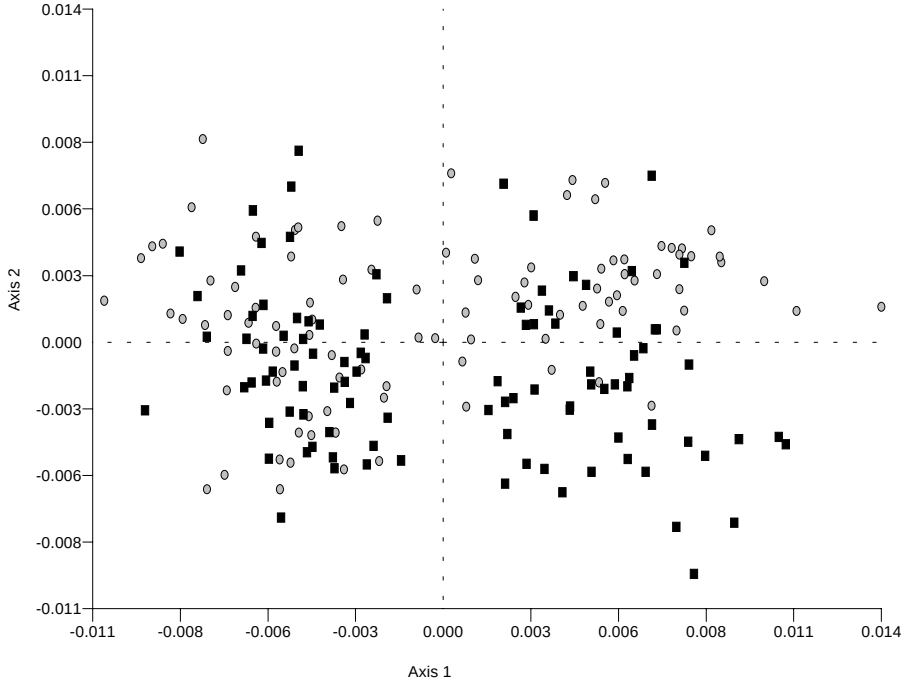

Fig. 7: multivariate analysis of the main components for comparison of normal erythrocytes (■) (n = 100) and erythrocytes parasitized (●) (n = 100) by *Hepatozoon philodryasi* of *Philodryas patagoniensis*. The values presented on the ordinate and abscissa axes represent the largest amount of variation in the data set.

We also observed different patterns when we analyzed the changes induced in the erythrocytes by the parasites. We observed that all parasites induced changes, mainly in erythrocyte length, as well as nuclear flattening (Table II) and the changes induced by *H. cyclagrasi* were more pronounced, with alterations of the erythrocyte and its nucleus in terms of all of the parameters studied. *H. migonei* induced deformity of the length and width of the erythrocyte but not in its area. This parasite also caused a complete distortion of the nucleus, changing its length, width, area, and position. *H. terzii* did not modify the area of the erythrocyte or of its nucleus, but induced significant modifications in their lengths and widths. *Hepatozoon* sp. of *C. durissus terrificus* induced changes involving the area and length of the erythrocyte, and also flattening of the nucleus. *H. philodryasi* was the species that least modified the erythrocyte. Even so, it altered its area and the width of the nucleus.

The results obtained by morphologic evaluation showed that, depending on the considered variable, such as, for instance, area and width of the parasite, we can separate five different species, whereas for another variable, such as length of the nucleus, we can differentiate only two populations (Table I). Since we believe that the individual analysis of a variable is not sufficient to characterize a species, we submitted all the data obtained for each parasite to multivariate analysis, a procedure that permitted us to characterize only three different populations (Fig. 2).

Current knowledge does not allow us to state whether *H. terzii*, *H. philodryasi* and *Hepatozoon* sp. of *C. durissus terrificus* are a single species or whether they are closely related different species. This characterization may be obtained after analysis of sporogonic and esquizogonic stages also with the use of molecular techniques for comparison of the respective DNAs. If it is proven that these are only one species, the use of morphometry, a simple and inexpensive technique, will be of fundamental importance for the characterization of *Hepatozoon* species. However, if subsequent studies demonstrate that the species are different, the technique used by us cannot be used for species characterization, but can only be considered as an additional methodology for the study of this group, associated with morphologic studies of other evolutionary forms of *Hepatozoon* spp.

In the present study we did not evaluate other evolutionary forms of the parasites, which could contribute to a differentiation of populations. However, Smith (1996) reported that *Hepatozoon* sp. exhibit a high degree of plasticity for many features and therefore, morphological and morphometric features of the oocysts stage must be statistically significant in order for species to be described. Recent studies have demonstrated that identification using molecular techniques may contribute to solve this problem (Wozniak et al. 1994, Smith et al. 1999).

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