In Vivo Differentiation of *Trypanosoma cruzi* - 1. Experimental Evidence of the Influence of Vector Species on Metacyclogenesis

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This work is dedicated to the late Prof. Dr Leonidas Deane for encouragement and his graceful invitation to share his office (Dept. of Entomology, Instituto Oswaldo Cruz) when our laboratory in Jacarepaguá became temporarily inaccessible for technical reasons.

Vector species has not hitherto been studied as influencing metacyclogenesis of Trypanosoma cruzi, *while the role of the parasite strain has been frequently stressed as of dominant importance in this process. In order to fill this gap in our knowledge, metacyclogenesis was monitored in nine triatomine species. The first part of this paper presents photographs of the main and intermediate parasite stages in each vector species studied. In the second part of the study the proportional distribution of all these forms, as seen in Giemsa stained smears is summarized, thus providing an opportunity to analyze both: the length of time between the ingestion of the blood trypomastigotes and the appearance of metacyclic forms and the rates of developmental stages leading to these latter. The most remarkable observation was that metacyclogenesis rates* in vivo *appear to be vector dependent, reaching 50% in* Rhodnius neglectus, *37% in its congener* R. prolixus *and being dramatically lower in the majority of* Triatoma *species (5% in* T. sordida, *3% in* T. brasiliensis *and 0% in* T. pseudomaculata*) at the 120th day of infection. These observations suggest that through screening of different vector species it is possible to find some that are capable of minimizing or maximizing metacyclic production.*

Key words: *Trypanosoma cruzi* - invertebrate host - vector species - intermediate forms - metacyclic yields

Trypanosoma cruzi can be cultured in a variety of media. Several of those have been recommended for mass production of metacyclic trypomastigotes - the parasite stage initiating Chagas' disease in humans and animals.

Most of these media required undefined components like insect hemolymph (Wood & Pipkin 1969), *Rhodnius prolixus* body extract (Wood & Sousa 1976), or *Triatoma infestans* embryo cells (Lanar 1979). In 1982, Sullivan reported that cultured epimastigotes when transferred to GMA (Grace's insect tissue culture medium), supplemented with bovine serum yielded over 90% metacyclic forms. Nonetheless, the search for more simplified and chemically defined media, to attend the growing demands for large numbers of metacyclics necessary in studies of immunoprophylaxis against Chagas' disease, continued.

In 1985 Contreras et al. reported an artificial triatomine urine medium (TAU) supplemented with proline, this supporting the yield of 92% of metacyclics within 140 hours. Such metacyclics displayed some biological properties similar to these observed in trypomastigotes from bugs, as resistance to complement lysis and macrophage digestion, adherence of epimastigotes to a substrate prior to transition to metacyclics (Bonaldo et al. 1988, Gonzales et al. 1988) previously observed in the insect host (Zeledon et al. 1984, Boker & Shaub 1984). Nonetheless, tests destined to estimate the functional similarity between the cultured and the bug derived metacyclics, should precede their routine use in biochemistry and immunoprophylaxis of Chagas' disease.

Therefore the development of *T. cruzi* Y strain has been followed in nine different vector species, representing domestic, peridomestic, sylvatic and sylvatic with tendencies of colonizing human homes, as described by P.-Szumlewicz and Muller (1982).

In this paper we provide new information beyond that reported by the senior author in 1979

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and more recently by Schaub and Losch (1988) and by Schaub (1989). It discusses the role that the vector species plays in the metacyclogenesis. It also provides new concepts and guidelines to epidemiologists involved in control of the disease (to be published).

MATERIALS AND METHODS

This study is an extention of a previous one, in search of an experimental insect model for xenodiagnosis (P.-Szumlewicz & Muller 1982) and most of the material and procedures adopted have been described there.

However statements, giving the main points of schemes applied, are briefly described: the *T. cruzi* Y strain was used throughout; it was received from Dr Z Brener, FIOCRUZ, in 1974 and was maintained in albino mice (occasionally in triatomine bugs); this strain was used to infect guinea-pigs utilized to engorge starved fourth instar nymphs of the following vector species: *R. neglectus, R. prolixus, P. megistus, T. infestans, T. sordida, T. brasiliensis, T. rubrovaria, T. pseudomaculata* and *T. dimidiata* (all laboratory reared since the early 70's).

It seems appropriate to mention that the schizodeme pattern of the parasite remained unchanged, as demonstrated in 1988.

All bugs were examined at different times post infection (pi) by a single feed on the infected guinea-pigs, starting with four or 15 days pi (Table I). The technique of bug examination involved cutting up the very rear portion of the abdomen, pulling out the digestive tract and grinding it in two or three drops of physiological saline.

The chief advantage of using the suspension of the entire digestive tract is that all of the forms could be found in the population at the time of examination, regardless their development sites.

Since impression smears offer the best method of classifying the individual development stages of the parasite by conventional microscopy, these were prepared as follows: concurrently with the preparation of wet films for xenodiagnostic purposes, three drops of the suspension were transferred with an inoculating platinum wire loop (3mm in diameter) on a clean slide. An equal volume of dog serum was added, mixed thoroughly and spred evenly. Two to five smears per bug were prepared depending on the density of parasite population in the suspension.

Such smears were dried and left overnight in an incubator at approximately 28°C. Subsequently, they were covered with May-Grunwald for 3 min. A similar volume of 1% $NaHCO_3$ in water was added and mixed with a capillary pipette. The fluid was removed after 1 min and the smear left for 1 hr in Giemsa' solution (30 drops per 10 ml of water) and washed thereafter in running tap water. Organisms were recorded on two or more smears with a Wild M20 photomicroscope using an oil immersion objective (1,000x).

The infected bugs were fed at biweekly intervals on chickens, but were never examined the week after engorgement. The reason for the former was the experimental evidence of the important part played by additional food given to bugs from xenodiagnosis on the parasite population kinetics, as observed by P.-Szumlewicz et al. (1988). The reason for the latter was that organisms uncovered in Giemsa stained smears were somewhat blurred by the excess of blood in the digestive tube, thus making the distinction of the different morphological stages more difficult if not impossible.

RESULTS

Morphological identification of the parasites - The nomenclature used in the identification of developmental stages observed herein was based on Hoare and Wallace (1966) and Pan (1968). It was extended to transitional forms from round stages to young epimastigotes and transitional forms giving rise to metacyclics, as discussed previously (P.-Szumlewicz 1979). Round or oval stages composed of the classical amastigotes and spheromastigotes are presented in Fig. 1. These follow the structure described previously by Pan (1968). *Epimastigotes*: organism elongated in shape with a kinetoplast anterior or beside the nucleus are shown in Fig. 2. These, irrespective of size and shape, multiplied mostly by binary equal and unequal fission, are shown in the upper part of Fig. 3 (1-13). The produced daughter cells (14-28) represent the intermediate forms leading from epimastigotes to metacyclics. *Trypomastigotes*: bloodstream or metacyclic ones are illustrated in Fig. 4. The latter derived from the binary equal fission of epimastigotes (1-13) and their filial cells (14-28) seen in Fig. 3. Although our reasoning is not completely free of imagination or speculation, it is sustained by the abundant transitional forms with intermediate characters between those of epimastigotes and metacyclics seen in the lower part of Fig. 3 (14-28). Less numerous were those from binary unequal division of epimastigotes (Fig. 5, 4-6), where one of the daughter cells represent a fully developed metacyclic, while the other kept still the morphological structure of epimastigotes. Nonetheless, this type of epimastigotes in fission represents one of the pathways leading to the production of metacyclics.

It seems appropriate to mention that on rare occasions metacyclic forms were observed that appeared to be dividing (Fig. 5, 7-16). This phenomenon occurred seldom and has been reported

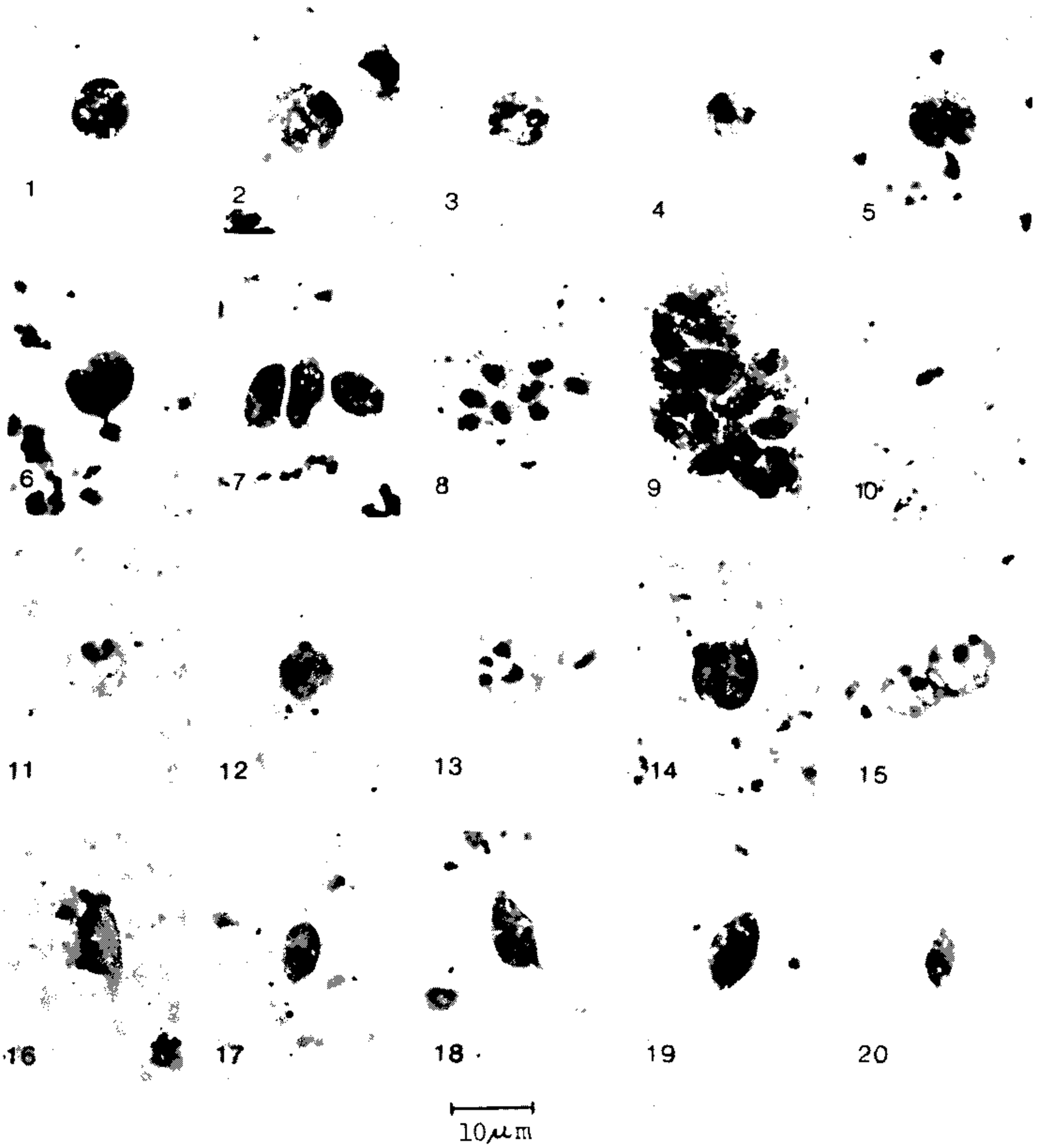

Fig. 1: photographs (1,000x) of round or oval stages found in Giemsa stained smears prepared from the entire digestive tract of *Triatoma* bugs, from four to 15 days after a single feed on guinea-pigs with an acute infection by *Trypanosoma cruzi* Y strain. *Amastigotes*: definite (1,2); in binary fission (3,4); leading to young epimastigotes (5-7); a mass of agregated amastigotes (9). *Spheromastigotes*: definite (10, 11); in binary fission (12-15); daughter cells in transition to epimastigotes (17-20). Both of these stages are uniform in shape and size, the mean diameter being 7.8 ± 1.0 μm. They differ with regard to the flagellum which is usually absent in the amastigote, when present it is short and located within the citoplasmic boundary, while it is free but motionless in the spheromastigote, as described in the literature. Both multiply by binary division and reversed to young epimastigotes (5-7 and 17-20). These two forms appear simultaneously during the transition of the blood trypomastigotes to the metacyclics and are essentially the same stage according to Pan (1968) and others. They are here combined and named round forms in transition; (8, 16) represent masses of organoids derived from desintegrated round forms, that usually occur in clusters.

also in cultures (Camargo 1964); it probably contributes little to the total production of metacyclics.

Quantitative determination of all morphologic types involved in the metamorphosis of blood trypomastigotes into metacyclic trypomastigotes - The comparative development of *T. cruzi* Y strain in nine different vector species is summarized in Table I.

Starting with four or 15 days after the infective meal, Giemsa stained smears of the contents from the alimentary tract of the bug were examined at biweekly intervals until 60 days and thereafter

TABLE I

Morphological types (%) of *Trypanosoma cruzi* in the digestive tract of the vector at different times after a single feed on infected mammals

Days pi	No. bugs	Forms exam. [a]	Round forms trans.	Epimastigotes		In trans.	Metacyclics
				Definite	Div.		
Rhodnius neglectus							
4	2	267	63.7	26.2	0.7	0	0
8	2	498	37.9	53.6	1.4	0	0
15	5	837	8.4	77.4	7.5	4.9	0.1
30	6	1036	0	65.6	10.2	15.0	5.6
45	5	1057	0	81.5 (79±15)	5.3	7.1	5.4 (6±4)
60	4	1003	0	67.3 (68±12)	9.2	17.2	2.5 (2±2)
90	4	721	0	33.4 (34±22)	7.2	32.9	24.5 (23±10)
120	5	1085	0	34.8 (31±15)	7.4	6.8	50.6 (51±10)
150	5	879	0	36.3 (34± 8)	5.2	14.4	43.7 (42±16)
180	6	1025	0.1	44.6 (51±27)	11.1	6.9	37.2 (32±21)
210	5	1134	0	28.0 (30± 9)	12.1	9.0	50.8 (48±15)
240	6	1565	0	46.3 (48±11)	8.4	14.7	30.4 (23±12)
300	1	478	0	61.1	11.5	3.8	23.6
R. prolixus							
4	2	85	68.2	4.7	0	0	0
8	2	197	52.3	27.4	0	0	0
15	4	472	24.4	39.2	3.4	3.6	0
30	5	710	0.3	79.7	4.8	9.2	2.3
45	4	427	0	78.0 (82±11)	5.2	14.8	1.9 (2±2)
60	3	298	0	65.1 (62±18)	9.1	23.8	1.3 (1±1)
90	4	660	0.1	33.2 (33±20)	7.3	20.2	37.9 (38±18)
120	3	455	0.2	50.3 (23±27)	4.8	13.0	30.3 (47±23)
150	5	127	0	52.0 (62±29)	3.1	21.3	22.8 (18±19)
180	3	299	0	68.0 (62±10)	5.0	13.4	13.7 (9±9)
210	3	736	0	39.3 (41± 8)	14.3	8.7	37.8 (35±12)
240	1	43	2.3	65.1	4.7	9.3	18.6
Panstrongylus megistus							
4	3	183	42.1	38.2	2.7	0	0
8	2	283	21.2	71.4	1.4	0	0
15	4	656	23.0	67.8	1.4	2.4	0
30	5	606	0.2	86.3	1.7	8.3	0
45	5	734	1.2	86.2 (89± 8)	2.0	3.8	2.0 (3±3)
60	4	807	0.1	81.0 (80± 9)	7.6	9.8	1.4 (1±2)
90	4	812	0	56.2 (54±10)	9.4	17.6	16.1 (17±7)
120	6	1064	0.5	60.0 (56±20)	8.2	11.8	18.0 (19±16)
150	4	1057	0	52.5 (57±18)	9.2	10.8	27.2 (22±19)
180	4	840	0	65.1 (63±22)	4.0	3.8	26.7 (28±17)
210	5	1484	0	59.0 (44±17)	9.3	6.1	24.5 (23 ±9)
240	6	1175	0.2	69.9 (70±10)	10.1	8.9	10.3 (8± 5)
Triatoma infestans							
4	2	211	67.3	18.5	0.5	0	0
8	3	357	28.6	52.1	0.8	0	0
15	2	584	16.4	77.9	1.9	2.7	0
30	3	872	15.0	68.2	3.2	4.5	0.2
45	4	734	18.5	61.9 (56±19)	4.8	8.6	1.5 (1± 1)

cont.

Days pi	No. bugs	Forms exam. [a]	Round forms trans.	Epimastigotes		In trans.	Metacyclics
				Definite	Div.		
60	3	352	17.6	68.8 (67± 8)	3.4	4.5	0
90	3	705	0.1	55.0 (62±21)	4.5	33.2	6.1 (6± 2)
120	2	480	0.4	71.9 (71±31)	6.0	5.2	16.0 (17±23)
150	3	569	0	38.8 (43±26)	4.7	13.7	42.0 (22±24)
T. sordida							
15	1	92	14.1	79.3	2.2	3.3	0
30	2	249	0	71.9	3.2	16.5	0
45	2	310	1.2	85.8 (87±11)	7.1	4.2	0
60	2	323	0	67.5 (67± 4)	6.5	24.5	1.5 (2± 1)
90	2	356	0.3	77.8 (78± 2)	9.0	9.0	3.0 (3± 4)
120	2	475	0	81.7 (79±14)	4.2	8.2	5.7 (6± 2)
150	2	327	0	86.2 (85± 5)	2.8	5.5	4.3 (5± 4)
180	2	452	0	56.4 (57±14)	10.2	10.0	23.0 (22±13)
210	2	684	0	80.3 (80± 5)	4.8	8.2	5.4 (5± 3)
240	2	417	0	62.4 (63± 2)	10.6	19.7	7.2 (7± 2)
T. pseudomaculata							
15	1	229	45.4	37.6	0.9	0	0
30	2	318	0.6	81.8	3.1	8.5	0.6
45	2	317	0.9	82.0 (82± 5)	6.3	9.1	0
60	2	300	0	95.0 (95± 1)	2.7	1.0	0
90	2	334	0	73.1 (73± 6)	5.7	12.6	1.2 (1± 1)
120	2	588	0	89.8 (92± 7)	2.4	6.3	0
150	2	265	0	78.1 (71±27)	2.3	10.6	3.0 (4± 4)
180	3	574	1.4	91.1 (91± 2)	4.5	1.1	0.4 (1± 1)
210	3	632	0.3	85.4 (71±12)	3.6	3.0	1.9 (3± 5)
240	2	593	0	52.3 (44±39)	8.6	21.9	16.4 (10± 3)
300	2	420	0	93.3 (93± 1)	2.4	3.8	0.2 (1± 1)
T. brasiliensis							
15	2	537	9.5	84.7	3.9	0.2	0
30	2	315	0	72.1	2.9	19.0	0.9
45	2	374	0.8	72.2 (73± 9)	9.6	9.1	4.5 (4± 5)
60	2	303	0	50.8 (51± 4)	11.2	34.3	2.7 (3± 0.1)
90	2	320	0	71.3 (73± 6)	6.3	14.1	7.2 (7±10)
120	2	740	0	79.1 (80± 3)	4.1	12.0	3.2 (2± 2)
150	2	[b]10	-	-	-	-	-
180	2	326	0	84.7 (80± 8)	5.5	8.6	0.6 (0.5±0.5)
T. rubrovaria							
15	2	201	5.5	89.0	2.5	0	0
30	2	81	3.7	77.9	3.7	1.9	0
45	2	334	0	88.6 (89± 3)	5.1	6.3	0
60	2	302	0	49.0 (46±12)	4.0	34.8	10.9 (8±10)
90	2	360	0	71.7 (72± 9)	7.5	18.1	1.9 (2± 1)
120	3	1054	0	65.8 (67± 4)	3.5	3.2	27.5 (27± 1)
150	2	314	0	86.6 (90± 6)	1.6	1.6	9.9 (7± 6)
180	3	338	0	67.5 (67±18)	1.8	3.9	26.9 (28±18)

cont.

Days pi	No. bugs	Forms exam. [a]	Round forms trans.	Epimastigotes		In trans.	Metacyclics
				Definite	Div.		
T. dimidiata							
15	1	[b] 5	-	-	-	-	-
30	2	64	0	48.4	3.1	0	0
60	1	320	0	51.3	2.5	44.7	1.2
90	1	325	0	44.6	9.5	18.5	26.4
120	1	121	0	81.8	0	13.2	5.0

The total number of developmental stages found in smears (2 to 5) from individual bugs (1 to 6) with heavy loads of parasites, as seen in wet films from the same insects that were used for xenodiagnostic purposes (P.-Szumlewicz & Muller 1982). These numbers form the samples of stages (column 3) for the evaluation of the five main morphological types shown in this table. [a]: total of forms found in smears from individual bugs more infected; [b]: number of forms insuficient for evaluation of rates. In brackets means and SD. Abreviations: pi = post infection; exam = examined; trans = transition; No. = number.

TABLE II

Relative frequency of epimastigotes and metacyclic trypomastigotes in varying vector species at different times after a single feed on infected mammals

Vector	*Rhodnius neglectus*	*Rhodnius prolixus*	*Panstrongylus megistus*	*Triatoma sordida*	*Triatoma pseudomac.*
Days pi	[a] Epimastigotes				
45	86.8 (0)	83.2 (0)	88.2 (1.2)	92.9 (1.2)	88.3 (0.9)
90	40.6 (0)	40.5 (0.1)	65.6 (0)	86.8 (0.3)	78.8 (0)
210	40.1 (0)	53.6 (0)	68.3 (0)	85.1 (0)	89.0 (0.3)
	Metacyclics				
45	5.4 (7.1)	1.9 (14.8)	2.0 (3.8)	0 (4.2)	0 (9.1)
90	24.5 (32.9)	37.9 (20.2)	16.1 (17.6)	3.0 (9.0)	1.2 (12.6)
210	[b] 50.8 (9.0)	37.8 (8.7)	24.5 (6.1)	5.4 (8.2)	1.9 (3.0)

Data extracted from Table I. Numbers in parenthesis indicate percentage of intermediate stages (upper part) between round forms and epimastigotes and (lower part) between epimastigotes and metacyclics. [a]: epimastigotes in division included. [b]: the first peak of a similar value (50.6) occurred at 120 days pi.

monthly or bimonthly until the stock of infected insects became exhausted, between 120 and 180 days in four species and between 240 and 300 days in the remaining ones.

The parasite population, uncovered at the fourth day after the meal on infected guinea-pigs, was mostly of transition forms (42.1 to 68.2%) from the round stages to epimastigotes. The relative proportion of these forms decreased steadily to nil between 15 and 30 days pi, and reappeared sporadically during subsequent periods at very low rates in all vector species examined, with the exception of *T. infestans* (Table I), which demonstrated over 17% of these forms as late as 60 days pi. This latter observation requires confirmation; consequently *T. infestans* was excluded from further observation made on the remaining eight vector species.

The dramatic decrease of the transition forms derived from round ones (Table I) was followed by the increase in number of epimastigotes, these, in turn, became dominant in the total parasite population found after the eighth day post infection and prevailed untill the end of the experiments. The few instances in which metacyclics were found to be the dominant stage occurred in *R. neglectus*.

There were intervals when epimastigotes, intermediate forms between them and well defined metacyclics were the only morphologic types found, transitional forms from round stages being absent,

Fig. 2: representative examples of epimastigotes seen in eight vector species: *Rhodnius prolixus* (1,2); *R. neglectus* (3,4); *Panstrongylus megistus* (5,6); *Triatoma infestans* (7,8); *T. brasiliensis* (9,10); *T. sordida* (11,12); *T. pseudomaculata* (13,14); *T.rubrovaria* (15,16). In contrast to the uniformity in shape and size observed in their predecessors - the round forms - the bizarre epimastigotes forms found in *P. megistus* (6) or *T. sordida* (11) raises questions on their source and significance. So does also the wide scatter of intra and interspecific dimensions from 24.8 ± 4.7 µm to 52.6 ± 4.6 µm, the intermediate being 41.7 ± 4.6 µm. The former is well exemplified by *R. neglectus* (3,4) and *T. pseudomaculata* (13,14), the latter by *T. brasiliensis* (9,10), *T. sordida* (11,12), *T. rubravaria* (15,16). So far available evidence indicates that very long organisms appear sporadically in the two *Rhodnius* species, *P. megistus* and *T. infestans*, while in *T. brasiliensis* (9,10), *T. sordida* (11,12) and *T. rubrovaria* (15,16) they exceed sometimes very short and medium size organisms, 24.8 ± 4.7 µm and 41.7 ± 2.9 µm, respectively (free flagellum included in dimensions).

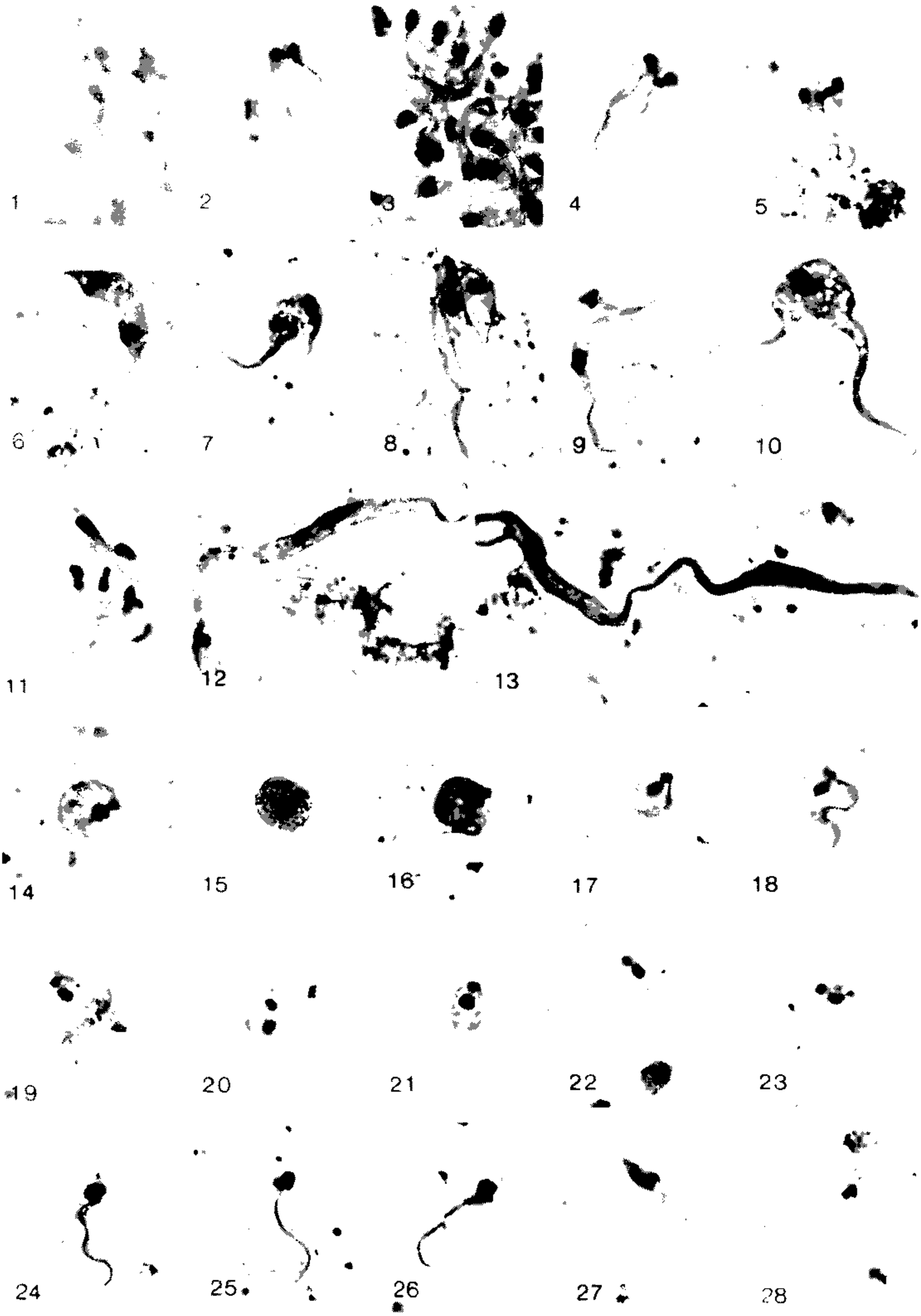

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TABLE III

Data suggesting that metacyclogenesis rate is vector species dependent

Vector	No. forms exam.	% round forms	% epimast.	% epimast. in trans.	% matacyc.
Rhodnius neglectus	6509	0,02	45.8±6.69	14.2±9.84	39.5±9.83
Rhodnius prolixus	2320	0.13	57.8±10.84	14.3±5.32	26.8±9.22
Panstrong. megistus	6432	0.11	68.8±5.59	9.8±4.83	20.5±6.16
Triatoma sordida	2711	0.04	81.1±8.29	10.1±4.92	8.1±6.78
Triatoma pseudomac.	2986	0.33	82.8±11.51	9.2±7.59	3.8±5.70

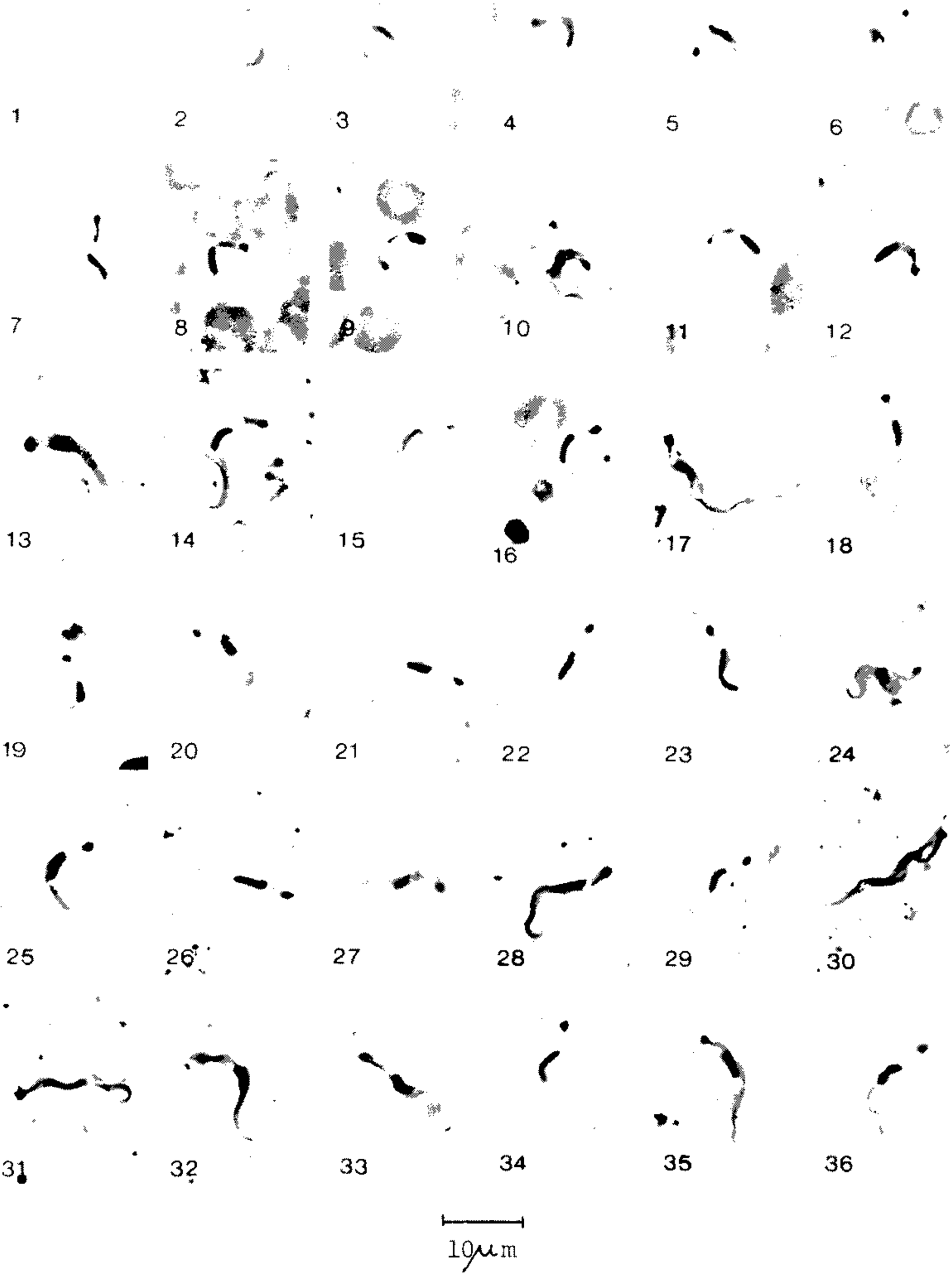
Results are based on overall data recorded six times within a period from 90 to 240 days pi, as seen in Table I. A statistycal comparison showed that the proportion of definitive epimastigote (column 4) and fully developed metacyclic (column 6) derived from *Trypanosoma cruzi* Y strain varied significantly ($p < 0.01$) in 10 different combinations of vector species. Among epimastigotes in transition to metacyclics (column 5) the proportions varied significantly ($p < 0.01$) in six combinations only. No significant variation occurred in the remaining four (*R. neglectus* x *R. prolixus; P. megistus* x *T. sordida; P. megistus* x *T. pseudomaculata* and *T. sordida* x *T. pseudomaculata*).

as indicated by zeros in Table I and also seen in brackets in Table II (upper part). This Table contains data derived from the preceding one. Their grouping separately contribute to a better understanding of the kinetics of the increasing metacyclics and decreasing epimastigotes as proportions of the totalities of parasite forms examined (column 3, Table I).

Mature metacyclics made their appearance in the alimentary tract of the bug within 30-60 days pi in all species examined, except *R. neglectus* in which they became perceptible as early as 15 days pi (Table I).

At 45 days pi the proportion of epimastigotes varied from 83.2 to 92.9% in the five vector species under consideration in Table II. At 90 days there was a dramatic fall in the proportion of epimastigotes in *R, neglectus, R. prolixus* and *P. megistus* (40.5 to 65.6%) which coincided with the increased proportions of metacyclics (16.1-37.9%) in these species (Table II). In contrast, only low proportions of metacyclics were found in *T. sordida* (3%) and in *T. pseudomaculata* (1.2%) in spite of relatively high rates of transition forms (9.0-12.6%). At 210 days pi the proportion of mature metacyclics reached 50.8% in *R. neglectus*, comparable with that observed in this species on day 120 pi. It remained unaltered (37.8%) in *R. prolixus*. This value was first observed in this species 90 days pi. In *P. megistus* it was 24.5%, slightly

Fig. 3: the upper part of this figure depicts multiplying epimastigotes (1-13) in eight different vector species: *Rhodnius prolixus* (1), *R. neglectus* (2,3), *Panstrongylus megistus* (4,5), *Triatoma infestans* (6,7), *T. sordida* (8,9). Most of these paratises suggest they undergo equal binary division. So does the epimastigote form found in *T. brasiliensis* (10) which also shows that the organoid and citoplasmic division is not always synchronous. In *R. neglectus* (3), and *T. rubrovaria* (11) the parasites seem to exibit the seldom occurring multiple equal or unequal division. A warning against erros in defining this kind of forms is based on the finding of fully devoloped metacyclics attached to epimastigotes, as seen in 2 and 11 which may not be the product from their division. This part of the figure depicts also the very long epimastigotes (56.6 ± 4.6 μm), suggesting binary division (12,13). In 12, one of the daughter cells is still an epimastigote while the other is already in transition.The former will probably devide anew, producing daughter cells that might also multiply thus contributing to the large proportions of epimastigotes present in the bug throughout its relatively long life span. In 13, the position of the organoids is unclear and will probably desintegrate (see Discussion). The bottom microphotographs (24-28) illustrate the forms that might be ahead of time in transition and approaching the ultimate step to complete metamorphosis. The intermediate stages (14-23) represent forms in transition from epimastigotes to metacyclics. They will probably require more time to reach the slender shape and the length (19.0 ± 3.0 μm) characteristic for metacyclics (Fig. 4). The round structures (14-16) in transition to metacyclics (as based on the post nuclear kinetoplast, the flagellum arising near it growing over the surface of the body, or encircling it and emerging on the opposite site, have a provocative similarity to the spheromastigote stressed by Brack as the intermediate link to the metacyclic (see Discussion). In our opinion this might be the product of binary fission of the bizarre epimastigote seen in Fig. 2 (6,8,11).



(see legend in next page)

lower than the peak of 27.2% registered 150 days pi (Table I).

Under the same experimental conditions the two *Triatoma* species examined presented poor differentiation, the proportion of epimastigotes remained practically unaltered within the period of observation (45 to 210 days).

It is interesting to note that there was not observed continuous raise in the metacyclic rates in our experiments, this being well exemplified in four species representing three different genera: *R. neglectus*, *R. prolixus*, *P. megistus* and *T. rubrovaria* (Table I).

Experiments aimed to determine the stability of metacyclic rates for each species, over a long duration of time (with more than one year), and the possible causes involved are currently in progress.

Data summarized in Table III aimed to show the possible influence of the vector species in the development of *T. cruzi*, mainly in the metacyclogenesis, by using overall data collected during this investigation. The advantage of such procedure has been previously mentioned by P.-Szumlewicz et al. (1990). Nonetheless, to avoid the loss of otherwise important information, occurring by pooling non manipulated data, we are in favor to include the oversized Table I, although the impression is that it may be unnecessary.

The practical significance of the emerged relationship between vector species and metacyclogenesis *in vivo* needs further research using other *T. cruzi* stocks (experiments in progress).

DISCUSSION, CONCLUSIONS, PROPOSALS

As mass methods described to increase differentiation of bloodstream trypomastigotes *in vitro* multiplied, the vector by itself was hardly remembered, although its involvement in the differentiation of blood trypomastigotes is not novel (Wood & Pipkin 1969, Wood & Sousa 1976, Lanar 1979, Sullivan 1982).

The natural host of *T. cruzi*, influencing its differentiation did not attract researchers, probably because the metacyclic yield *in vivo* is rather low. But, since the cultured metacyclic must be viewed in the perspective of its usefulness in the development of an immunologic system against the infection by *T. cruzi*, it is absolutely essential to compare its validity with that of its *in vivo* developed counterpart.

The uniqueness of this paper is the volume of data accumulated in studying development of *T. cruzi* in nine vector species. We recorded all morphological stages found in the entire digestive tract. This approach may be less ambitious than the determination of the developmental stages separately in their sites of origin. However, it appears to us more realistic, particularly in a study covering the evolution of metacyclics simultaneously in nine vector species.

The data summarized in Tables and Figs provide an unprecedent opportunity to analyze both: the parasite yield of each developmental stage of the parasite and the length of time for appearence of the metacyclics. It will be well keeping in mind that quantitative estimates here described offered a better idea of the successive transformation of earlier to later forms. Thus, the development of *T. cruzi* in their natural hosts is supplemented with data, beyond these discussed in the past (P.-Szumlewicz 1979).

It is perhaps appropriate to mention that parasite populations, observed in bugs at successive days during the first week pi, consisted of decreasing numbers of slender and broad trypomastigotes, short and stumpy intermediate forms between them and amastigotes, increasing numbers of the latter and fully developed epimastigotes. Practically all mice inoculated intraperitoneally with this blend died (197 of 203 tested) within a period from 9 to 17 days after inoculation (unpublished). Such short and stout trypomastigotes accompanied by amastigotes observed in the intestinal tract of bugs the first days after feeding on donors with acute infections by *T. cruzi* Y strain, appear in fact to be preliminary stages of extracellular differentiation of blood stream trypomastigotes within the vertebrate host (Andrews et al. 1988).

Fig. 4: for comparative purposes the first two horizontal lines showed blood trypomastigote in mice (1-6) and in guinea-pigs (7,12). Metacyclics seen in nine different vectors species fed once in guinea-pigs: *Rhodnius prolixus* (13-15); *R. neglectus* (16-18); *Panstrongylus megistus* (19-21); *Triatoma infestans* (22,23); *T. brasiliensis* (24-26); *T. sordida* (27,29); *T. pseudomaculata* (30,31); *T. rubrovaria* (32-34) and *T. dimidiata* (35,36). The organisms were generally slender in shape, being similar to the slender bloodstream trypomastigotes from infected mice (1-6). Total length of metacyclics (free flagellum included) was 19 ± 3.0 µm while it was 18± 1.7 µm and 19.9 ± 2.8 µm in trypomastigotes from guinea-pigs and mice, respectively. The nuclear index-ratio of distance from the middle of the nucleous to the anterior and posterior end of the body (the flagellum not included) was 0.6 in the metacyclics, while it was 0.9 and 0.8 in blood trypomastigotes from guinea-pigs and mice, respectively. This lower index in metacyclics was due to the different position of the nucleous, entirely located, in the posterior part of the metacyclic, while it is near the middle of the body in blood trypomastigotes. The biometrical variations described for metacyclics could not be considered vector species specific.Consequently the mensuration of the metacyclics will be of dubious help in attempt to distinguish the vector species, which might be involved in the transformation of a blood trypomastigotes in metacyclics.

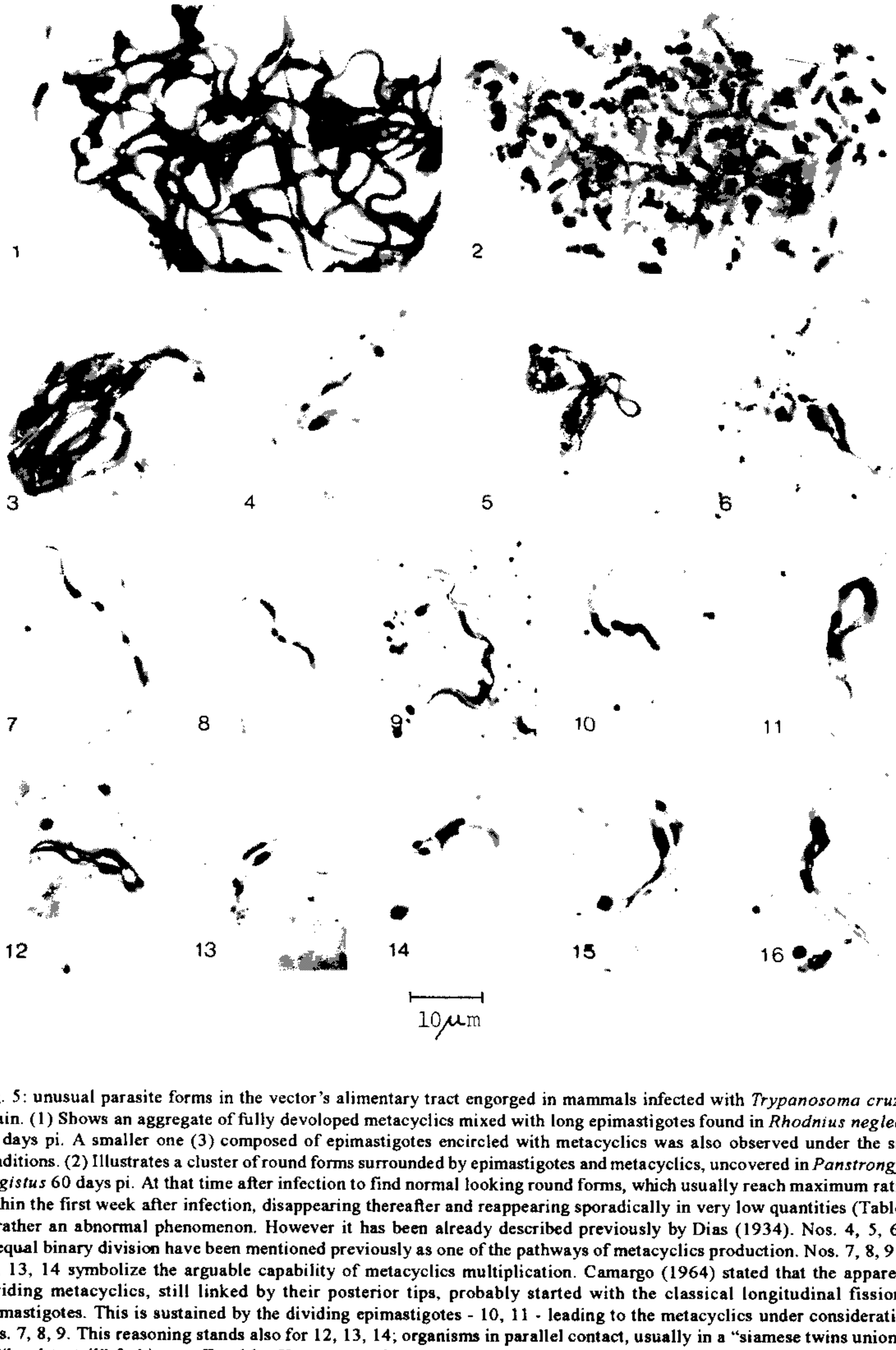


Fig. 5: unusual parasite forms in the vector's alimentary tract engorged in mammals infected with *Trypanosoma cruzi* Y strain. (1) Shows an aggregate of fully devoloped metacyclics mixed with long epimastigotes found in *Rhodnius neglectus* 90 days pi. A smaller one (3) composed of epimastigotes encircled with metacyclics was also observed under the same conditions. (2) Illustrates a cluster of round forms surrounded by epimastigotes and metacyclics, uncovered in *Panstrongylus megistus* 60 days pi. At that time after infection to find normal looking round forms, which usually reach maximum ratings within the first week after infection, disappearing thereafter and reappearing sporadically in very low quantities (Table I), is rather an abnormal phenomenon. However it has been already described previously by Dias (1934). Nos. 4, 5, 6, in unequal binary division have been mentioned previously as one of the pathways of metacyclics production. Nos. 7, 8, 9 and 12, 13, 14 symbolize the arguable capability of metacyclics multiplication. Camargo (1964) stated that the apparently dividing metacyclics, still linked by their posterior tips, probably started with the classical longitudinal fission of epimastigotes. This is sustained by the dividing epimastigotes - 10, 11 - leading to the metacyclics under consideration - Nos. 7, 8, 9. This reasoning stands also for 12, 13, 14; organisms in parallel contact, usually in a "siamese twins union" or in "head to tail" fashion, reffered by Hoare as conjugated cells. The possible predecessors of these latter could be the epimastigotes in division - 15, 16.

In 1968, Brack established that the spheromastigote through a process of unrolling and flagellar growth developed directly into a metacyclic. However, the experiment described to support this was not convincing. This author induced a single spheromastigote in each of the 37 *R. prolixus*, only one became infected, as shown by the mixture of epimastigotes and metacyclics found 42 and 60 days pi. But these latter could be the product of an unequal fission of epimastigotes in two organisms, a metacyclic either mature or in transition and the other still displaying a morphological pattern characteristic for epimastigotes, thus representing one of the normal pathways of metacyclogenesis (Fig. 5, 4-6). No evidence that links the spheromastigotes directly to the metacyclics was disclosed. On the contrary, the spheromastigotes stressed by Brack as the prestage of the metacyclic, has been found in no reasonable proportions to match the metacyclic rates found at 90 or more days pi. This author also suggested the possible existance of two parallel cycles, the spheromastigotes differentiating either directly to metacyclics, or indirectly through the epimastigotes. These latter according to the scheme outlined in the paper regress to round forms which gradually develop into metacyclics. This hypothesis cannot be accepted unreservedly, the major objection being the time when the forms are available; the round forms reached their highest level within the first week pi and disappeared, while the metacyclics became detectable after 30-60 pi in the presence of epimastigotes which rates varied from 60 to 90% approximately.

In our study, metacyclics made their appearance in the alimentary tract of the bug occasionally on day 15 pi. However, they were usually detected from 30 to 60 days pi, increasing slowly, bud did not grow continuously. They reach 50% in *R. neglectus*, 37% in *R. prolixus*, 27% in *P. megistus* and *T. rubrovaria* (Table I), followed by a decline which runs its course in a relatively short period, after which differentiation resumed growth, leading to a second peak, that compares well with the previous one. In *T. infestans* the metacyclic rates reached one of the highest levels (42%) registered in this study, but, at the time being it is out of consideration for reasons mentioned above (Results). Cyclic declines between two equal peaks occurred in all species which due to the availability of experimental bugs could be analyzed up to 240-300 days pi. Whether the last decrease of metacyclic rates at the end of experiments was temporary or due to aging of the infection or/and the host, could not be answered; our observations finished due to lack of experimental bugs.

So far we do not know the factor(s) regulating metacyclogenesis in the insect host. Neither do we know the elements which maintain the high rates of metacyclics in some species of bugs. But much the same is undergoing in metacyclogenesis *in vitro*; the reasons why substances, as reported by Wood and Sousa (1976) and Ross (1987), or electrolytes, as described by Osuna et al. (1990), which ordinary stimulate metacyclogenesis, when added in excess reduce the yield of metacyclics to the control level.

Our view on the eulogistic status ascribed by Brack to the spheromastigote as the prestage of the metacyclic has been revealed in 1979, and confirmed recently by Schaub (1988). This author, upon recording only 2% of spheromastigotes in the small intestine and 3% in the rectum of the bug, concluded that "the spheromastigote cannot have the important role in the life cycle of *T. cruzi* proposed by Brack".

On the other hand, the epimastigote was reduced by this author to a lower importance in the metacyclogenesis of *T. cruzi*. One has to admit that, had this stage occur in low numbers, it could have turned its presence as sporadic, anomalous and of minor importance. In contrast, in the present study the ratio of epimastigotes in the parasite population preveiled at any time of the bug infection, thus indicating that it is in fact the most common and persistent stage in the process of metacyclogenesis *in vivo*, suggesting that all the intermediate forms (Fig. 3, 14-28), leading to the formation of the metacyclics emerged from the epimastigotes which represent the unified field of characteristics derived from the blood trypomastigotes.

This takes us back to the very early concepts by Brumpt (1912), Dias (1934) and Camargo (1964), when the metacyclic was regarded as originated from the epimastigote. Then this paper seems to elucidate the long lasting dispute (Brener & Alvarenga 1976) related to the importance of the epimastigote as source of the infective stage of *T. cruzi* in the vector.

It is the first time that support to conclusions related to metacyclogenesis *in vivo* was commanded by quantitative records, which could explain this process in a manner that runs beyond the published disputes and speculations empiric in nature. Moreover, as intermediate forms and metacyclics appear in the invertebrate host we have been contemplating a possible acceleration of the formation of metacyclics when they emerge from the slender, short or medium size epimastigotes (Fig. 2, 1,4,14) and a retardation when they derive from the bizarre in shape or extremely long epimastigotes (Fig. 2, 6,8,11 or 9,10,12,15,16). We believe that these latter have to undergo more suc-

cessive divisions to reach the shape and size near that of metacyclics, thus increasing the time of their development. As for the destiny of the long epimastigotes we diverge from Brack (1968) and Souza (1984) who contend that they are aimless forms in metacyclogenesis, doomed to disintegrate. We rather agree with Schaub (1989) on the general approach toward the long epimastigotes, while diverging on some details. We support the existence of long multiplying epimastigotes; however, we believe that not all of their daughter cells will divide: the very long with a kinetoplast posterior or beside the nucleus will not divide, while the remaining (with characteristics of normal epimastigotes) will divide when necessary, thus contributing to the persistency of the epimastigotes in the bug. Our view gained ground because no examples of long transitional epimastigotes, suggesting division were found among the 45,758 organisms examined in this study.

Earlier workers did mention the existence of transitional or intermediate forms occasionally only. But the reason for their presence extends beyond occasional appearance in the life history of the parasite in the bug. Their presence is absolutely necessary to complete epimastigote differentiation into metacyclics. They were found *in vivo* by Boker and Shaub (1984) and Zeledon et al. (1984), and *in vitro* by Gonzales-Perdomo et al. (1988) and Bonaldo et al. (1988), in populations of differentiating epimastigotes, which adhere to a substrate before metamorphosis to metacyclics. They also accompanied the developed matacyclics exposed to purification by the selective lysis of epimastigotes (Nogueira et al. 1975). In case they function like the fully mature metacyclics, they will contribute to the increase of the infectious stages in the bug, and its capability to transmit the disease. Our reasoning in favor of such a possible joint effect of forms (last two columns in Table I) is sustained by Sher et al. (1983) and Contreras et al. (1985), showing that stage specific gene activation preceeding the ultimate morphological changes might speed up metacyclogenesis and increase the metacyclic rates (92%) within 16-24 hr and 12-140 hr, respectively.

By piecing results obtained together we came to the following conclusions: (1) had the spheromastigote been the prestage of metacyclic developed *in vivo*, as advocated by Brack, it had to be found in a proportion, at least, similar to that of uncovered metacyclics, but that was not the case (Tables I, II, III). Practically they stoped to exist, starting with day 15th pi, while epimastigotes and metacyclics continued until the end of the long lasting experiments, from 240 to 300 days pi (Table I); (2) with the revalidated knowledge related to the epimastigotes, the most controversial stage in the differentiation of blood trypanosomes *in vivo* since 1968, it is no longer necessary to defend it, as the main source of metacyclics. Any epimastigote can transform to a metacyclic as long as the vector, that constitute the biotope and ecological niche of the parasite, does not interfere with its differentiation; (3) the transitional stages between epimastigotes and the metacyclics (Fig. 3, 14-28) are with high probability the immediate predecessors of these latter. But the reason of their presence can extend beyond the life cicle of the invertebrate trypanosome, as mentioned above. They may also contribute to the increase of the infectious forms in the parasite population. Therefore it seems interesting to answer the question whether the surface antigen present on the epimastigotes can also be present on the immediate transition forms leading to metacyclics; (4) as for the metacyclics, the conclusion is that their rating *in vivo* compared poorly with that produced in their counterpart *in vitro*. This finding raise the question what makes the differentiation of blood trypanosomes a successful process *in vitro* around 92% of metacyclics (under certain conditions), as reported by Sullivan (1982), Sher et al. (1983), Contreras et al. (1985) versus a maximum of only 39.5 ± 9.83 (Table III) in this study.

It is well known that developmental stages of protozoan parasites are rapidly lyzed in fresh normal serum by means of alternative complement pathways (ACP). Therefore lysis may be, at least in part, responsible for poor metacyclogenesis in the invertebrate host. Scientists have also long suspected that the development of *T. cruzi* in its natural host could be associated with the presence of lectins.

In 1980, Pereira et al. reported that specific receptors for wheat germ agglutinins (WGA) were found in culture epimastigotes, whereas peanut agglutinins (PNA) were present exclusively in amastigotes. One year later the isolation of lectins from different portions of the alimentary tract of *R. prolixus* was reported (Pereira et al. 1981). These authors also mentioned that their preliminary experiments indicated the presence of lectin activities in the hemolymph of *Triatoma vitticeps, T. infestans, Panstrongylus megistus* and others. This finding made scientist to believe in their regulatory role as respect the metacyclogenesis *in vivo* (Sher & Snary 1982).

The quantitative data (Table II) suggest that the gradual increase of metacyclic rates coinciding with the decrease of epimastigotes might be the result of lectin activity in the insect. The presence of lectins, capable of binding *T. cruzi* stages at the sites of their emergence, makes the main

objective of this study - fast differentiation of epimastigotes and high metacyclic yields - completely unreal. Not exactly, as based on evidence accumulated for many months of observation, the conclusion that metacyclogenesis *in vivo* is vector species dependent is inescapable. Consequently, it may argue for success in finding an insect model capable to sustain high metacyclogenesis rates in a short time. This latter is dramatically long in bugs - several months versus 24 hr to six days in culture organisms and several days in mice.

Meanwhile research described came in touch with a subject that has been outside the scope of this study at its start. Namely, it has been insisted that harvesting of large numbers of the *in vivo* developed metacyclics is a precondition for integrating the *in vitro* produced metacyclics in the elaboration of immunochemical systems against Chagas' disease. However, as research unfolded, the vector, appeared to be the partner of undisputed major importance among elements regulating metacyclogenesis. Before drowing any rush conclusion, the results herein described indicate that some of the vector species are potentially able to limit human and animal infections with *T. cruzi* due to their incapability to favor the process of metacyclogenesis. Such is our answer to the question raised-why in certain endemic areas of the State of Piauí, Brazil, the indices of human infections are low as compared with those found in the bug population, mainly composed of *T. pseudomaculata* (to be published elsewhere).

Therefore, in attempts to delineate areas where future research on parasite-vector interaction may be most productive in minimizing infection rates in Chagas' disease, our strategy might be sumed up as follows: (1) seek information related to metacyclogenesis of different *T. cruzi* strains in the same vector; (2) search the factors influencing metacyclogenesis of the same *T. cruzi* strain in different vector species; (3) in contemplating another working system in search of an insect capable to produce large proportions of metacyclics, it seems interesting to apply to the vector some of the cues that have been successfully adopted to metacyclogenesis *in vitro*, like the exact pH recommended by Wigglesworth (1951), Fernandes et al. (1969), Ucros et al. (1983) or the right blend of electrolytes stressed by Osuna et al. (1990). This implies the use of bugs engorging directly from a source of blood. Such has been found to be a success by Garcia et al. (1984) in large scale rearing of vectors of Chagas' disease. Generally speaking, this allows to study the virtually unknown ecology the parasite finds upon moving from the vertebrate to the invertebrate host (Zeledon et al. 1977).

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