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Dynamic development of *Trypanosoma cruzi* in *Rhodnius prolixus*: role of decapitation and ecdysone therapy

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Abstract Decapitation and ecdysone therapy on the population dynamics of the *Trypanosoma cruzi* Dm28c clone in the stomach, small intestine and rectum of fifth-instar larvae of *Rhodnius prolixus* were investigated. Parasites were not found in the small intestine and rectum of decapitated insects after10 days post-infection (p.i.). Decapitated ecdysone-supplemented insects sustained the flagellate infection in both gut compartments. In the rectum, the population density of parasites increased 5-fold in ecdysone-treated decapitated larvae and 7-fold in control insects. Epimastigote forms dominated with 40–65%, intermediate stages and round forms varied over 10–35% in the stomach, small intestine and rectum in both insect groups. Low numbers of metacyclic trypomastigotes were observed in the stomach and small intestine of the control group and decapitated insects supplemented with ecdysone but, at 15 days p.i., this form of flagellate reached about 20% in the rectum of the control insects. In the entire gut, at 30 days p.i., 23% of parasites in the control group and 8% in the decapitated insects treated with ecdysone were found. These results indicate that a head factor, possibly the prothoracicotropic hormone from the brain which stimulates ecdysone production by the prothoracic glands, may act directly or indirectly to stimulate the development of epimastigotes and round forms of the parasite and that a single ecdysone treatment is not able to fully reverse metacyclogenesis in decapitated *R. prolixus*.

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Introduction

Chagas disease is one of the major endemic diseases in Latin America (Brener 1973). Triatomine vectors transmit *Trypanosoma cruzi*, the causative agent of the disease, during a blood meal on the vertebrate host (Chagas 1909). This flagellate displays different morphological and functional forms when alternating between vertebrate and invertebrate hosts. It also alternates between replicative stages (epimastigotes in the vector gut, amastigotes in mammalian cells) and infective, non-dividing stages (metacyclic trypomastigotes in the insects, which are deposited together with feces and urine, and bloodstream trypomastigotes in mammals; Brener 1973; Garcia and Azambuja 1991, 2000; Kollien and Schaub 2000). When the insect feeds on infected blood, the bloodstream trypomastigotes transform predominantly into epimastigotes, which colonize the whole gut and differentiate into metacyclic trypomastigotes in the rectum, a process known as metacyclogenesis (Brener 1973; Garcia and Azambuja 1991, 1997; Azambuja and Garcia 1997; Kollien and Schaub 2000).

Larvae of the hematophagous hemiptera *Rhodnius prolixus* undergo a head critical period (HCP) for moulting which is related to their feeding time (Wigglesworth 1934a, b, 1940). This period depends on the brain factor or brain hormone, termed the prothoracicotropic hormone (PTTH), which, due to its stimulatory effect on the prothoracic glands, leads to the release of the moulting hormone, ecdysone (Gilbert et al. 1980). Using decapitation and head transplantation experiments, Knoblock and Steel (1989) and Garcia et al. (1990) demonstrated that heads originating from 5th-instar larvae of *R. prolixus* donors removed 4–6 days after a blood meal, when implanted onto decapitated hosts, restored ecdysteroid levels which progressively diminish in headless insects.

Recently, Gonzalez et al. (1999) demonstrated that decapitation caused a decrease in trypanosome infection, while in converse experiments, transplantation of

heads obtained from donors during the HCP or ecdysone therapy re-established the parasite infection in *R. prolixus* larvae decapitated 1 day after feeding. Employing these bioassays, Garcia et al. (1990) and Gonzalez et al. (1999) tested the impact of the brain neuroendocrine system of *R. prolixus* on the progress of *T. cruzi* infection and demonstrated that decapitation and ecdysone therapy influence the total number of flagellates in the gut of the vector. Considering the feeding, heads obtained during the HCP and implanted onto hosts decapitated 1 day after feeding (before the HCP) restored the ecdysone level in the hemolymph, which progressively decreased in decapitated insects. Therefore, we focused this study on the dynamics of parasite infection of *T. cruzi* in the stomach, small intestine and rectum of decapitated and ecdysone-treated decapitated *R. prolixus*, through quantitative and qualitative analysis of different developmental forms.

Materials and methods

Insect and parasite

Rhodnius prolixus was obtained from a longstanding colony reared and maintained in the laboratory at 28 °C, as described by Azambuja and Garcia (1997). The *Trypanosoma cruzi* Dm28c clone, characterized as TC 1 group, zimodeme I, type III, lineage 2 and ribodeme II/III (Andrade 1974; Clark and Pung 1994; Tibayrenc 1995; Souto et al. 1996; Fernandes et al. 1999), was kept in NNN and brain heart infusion (BHI, Difco) media, as described by Garcia and Azambuja (1997).

Infection and counting of parasites

Fifth-instar larvae of *R. prolixus*, starved for 45 days after the last ecdysis, were allowed to feed on a membrane apparatus containing heat-inactivated citrated human blood and epimastigotes (3.0×10^6 parasites/ml) grown for 7 days in BHI medium (Garcia and Azambuja 1997). Less than 2% of metacyclic trypomastigotes were found in the culture medium at this time. At various intervals post-infection (p.i.), different gut compartments (stomach, small intestine, rectum) or the whole midgut tract of six insects was removed and gently homogenized in phosphate-buffered saline (PBS) at pH 7.2. Additional PBS was added to complete the homogenate volumes to 1,000 µl for the whole tract, 150 µl for each stomach, 20 µl for intestinal homogenates and 50 µl for rectal material. The number of parasites in each homogenate was determined using a Neubauer hemocytometer.

Different developmental forms of parasites

The percentage of different morphological stages of *T. cruzi* in the stomach, small intestine and rectum was evaluated using Giemsa-stained smears. After staining, 150–200 *T. cruzi* developmental forms from different gut compartments of each insect were classified according to Schaub (1989) in five classes: (1) round forms (RF) as amastigotes and spheromastigotes, (2) epimastigotes (E) with an anterior kinetoplast, (3) intermediary forms (IT) between epimastigotes (or spheromastigotes) and trypomastigotes with kinetoplast beside or behind (not subterminal) the round or elongated nucleus, (4) ring or onrolling forms (RF/E) and (5) long or short metacyclic trypomastigotes (T) with a subterminal kinetoplast. Figure 1 demonstrates the intermediate forms between spheromastigote and epimastigote or metacyclic trypomastigote and a division of such stages.

Endocrine manipulation

Fifth-instar male larvae were used in decapitation experiments. Since ecdysone titers are constant during the first 5 days after feeding (HCP) (Knoblock and Steel 1989; Garcia et al. 1990), the insects were decapitated on day 1 p.i. (before HCP) with a small razor and the wound was sealed with molten (45 °C) liquid wax. The success of the decapitation procedure was evaluated by checking for a withdrawal reflex after the application of delicate pressure to the legs with forceps. Insects that failed this test were rejected. The mortality after decapitation and recorded daily was less than 10%.

Ecdysone therapy

α-Ecdysone (Aldrich) diluted in ethanol:PBS (1:4) was added to the blood, resulting in a final concentration of 5 µg/ml, immediately before feeding. Control insects received a blood meal which contained the ethanol:PBS solvent but no α-ecdysone.

Results

Levels of *Trypanosoma cruzi* infection in different gut regions

After ingestion of about 3.6×10^5 parasites/insect, approx. 1.9×10^6 parasites/stomach developed within the initial 5 days p.i. in all groups (Fig. 2A), decreasing by about 99% within the following 5 days. No parasites were observed in the stomach of bugs of any group at 15 days p.i.

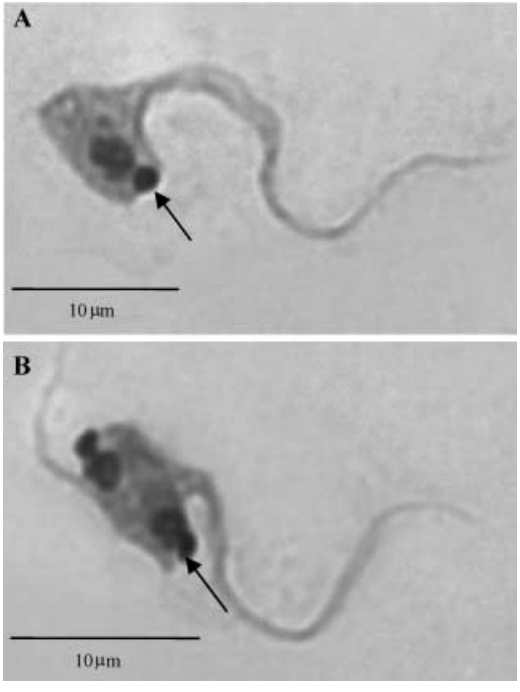

Fig. 1A, B Light micrographs of developmental stages of the *Trypanosoma cruzi* DM28c clone in the digestive tract of *Rhodnius prolixus*: intermediate forms between spheromastigotes and epimastigotes or metacyclic trypomastigotes (**A**) possessing the kinetoplast beside the nucleus, (**B**) division of such a stage

The small intestine was colonized by about 2.0×10^6 parasites at 5 days p.i. in all groups (Fig. 2B). In the controls, the infection remained at a high level during the following 10 days. However, no parasites were found in the small intestine of decapitated insects at days 10 and 15 p.i. (Fig. 2B). At day 5 p.i., decapitated insects treated with ecdysone sustained the flagellate infection at levels as high as those observed in the control insects. However, the parasite infection was 2.8×10^4 and 1.8×10^4 flagellates/small intestine at days 10 and 15 p.i., respectively (Fig. 2B).

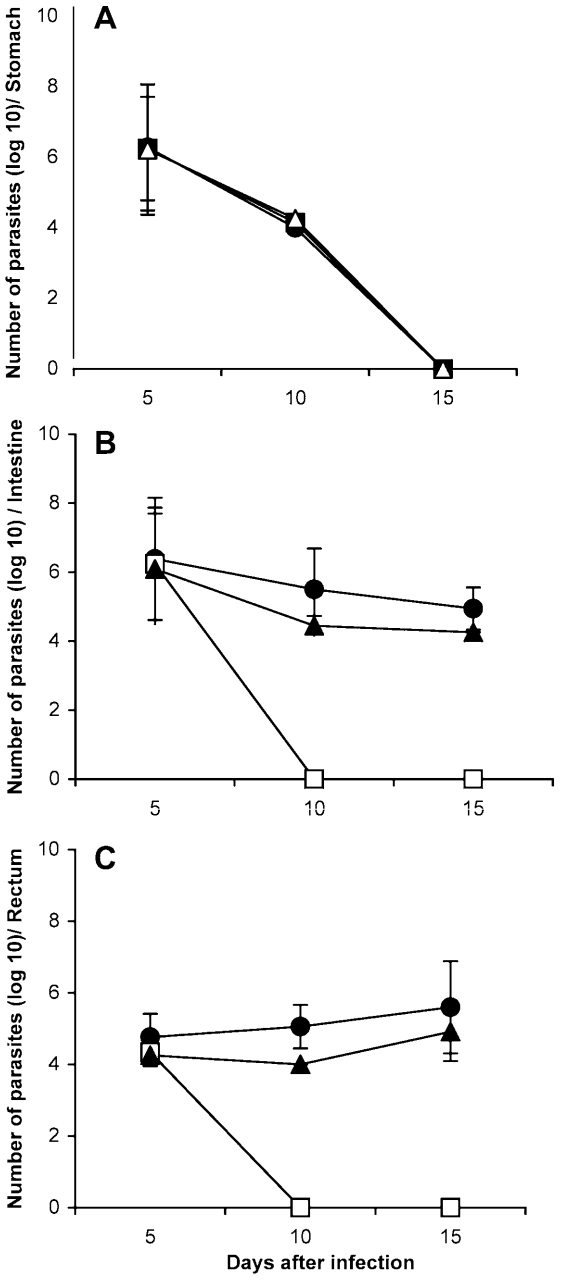

Fig. 2A–C Effect of decapitation and ecdysone therapy on the course of *T. cruzi* Dm28c clone infection in the stomach, small intestine and rectum of fifth-instar larvae of *R. prolixus*. Insects were fed on blood containing 3.0×10^6 epimastigotes/ml and then decapitated 1 day after feeding. Ecdysone-treated insects received a single dose of α-ecdysone in the blood meal (5 μg/ml). **A** Stomach, **B** small intestine and **C** rectum. *Black circles* control insects, *black triangles* ecdysone-treated decapitated insects, *white squares* decapitated insects. Each point represents the mean ± SD of parasites in the gut compartments of six insects

In the rectum, the trends of development were very similar to those of the populations in the small intestine (Fig. 2B, C): Parasites were not detected in decapitated insects 10 or 15 days p.i. and the development in control and ecdysone-treated decapitated insects followed the same profiles as the infection in the small intestine (compare Fig. 2B, C). In the rectum, at 5–15 days p.i., the parasite population density increased from 5.8×10^4 to 39.6×10^4 (7-fold increase) in controls and from 1.8×10^4 to 8.2×10^4 (5-fold) in ecdysone-treated decapitated insects.

Development of the different morphological forms of *T. cruzi*

In the stomach, at 5 and 10 days p.i., epimastigotes, intermediate forms and round forms of *T. cruzi* were the main forms detected in the controls, decapitated insects and ecdysone-treated decapitated larvae (Fig. 3). Epimastigotes always dominated with 40–60%, whereas the percentage of both the intermediate stages to metacyclic trypomastigotes and round forms varied over 20–35%. Low numbers of metacyclic trypomastigotes ($<10\%$) were observed in the stomach of control insects at 5 and 10 days p.i., while, in the groups of decapitated insects and ecdysone-treated decapitated larvae, this form of parasite was only found at 5 days p.i. (Fig. 3B, C).

In the small intestine epimastigotes, intermediate forms and round forms also predominated in all flagellate populations, possessing mean average levels of 45–65%, 20–35% and 10–30%, respectively (Fig. 3). The relative percentages of these morphological forms were similar in all groups. In controls and ecdysone-treated decapitated insects, these percentages were also similar at 10 and 15 days p.i., except that the low percentages of trypomastigotes appeared at 10 and 15 days after feeding in these insects (Fig. 3). The percentages of the metacyclic trypomastigotes never exceeded 2% in small intestine of individual bugs.

In the rectum, epimastigotes and intermediate forms to trypomastigotes were the most frequently observed developmental forms of parasite found in *Rhodnius prolixus* control larvae (Fig. 3A). Metacyclic trypomastigotes were detected in these larvae during the entire 15 days of the experiment, finally reaching nearly 20% (Student's *t*-test: $P<0.005$). At 5 days p.i., the composition of the populations was similar in all three groups of bugs, except for the occurrence of metacyclic trypomastigotes in the control larvae (Fig. 3). The same was evident in the comparison of the populations at 10 and 15 days p.i. However, ecdysone therapy of decapitated insects significantly increased the percentages of round forms, in the latter comparison, when

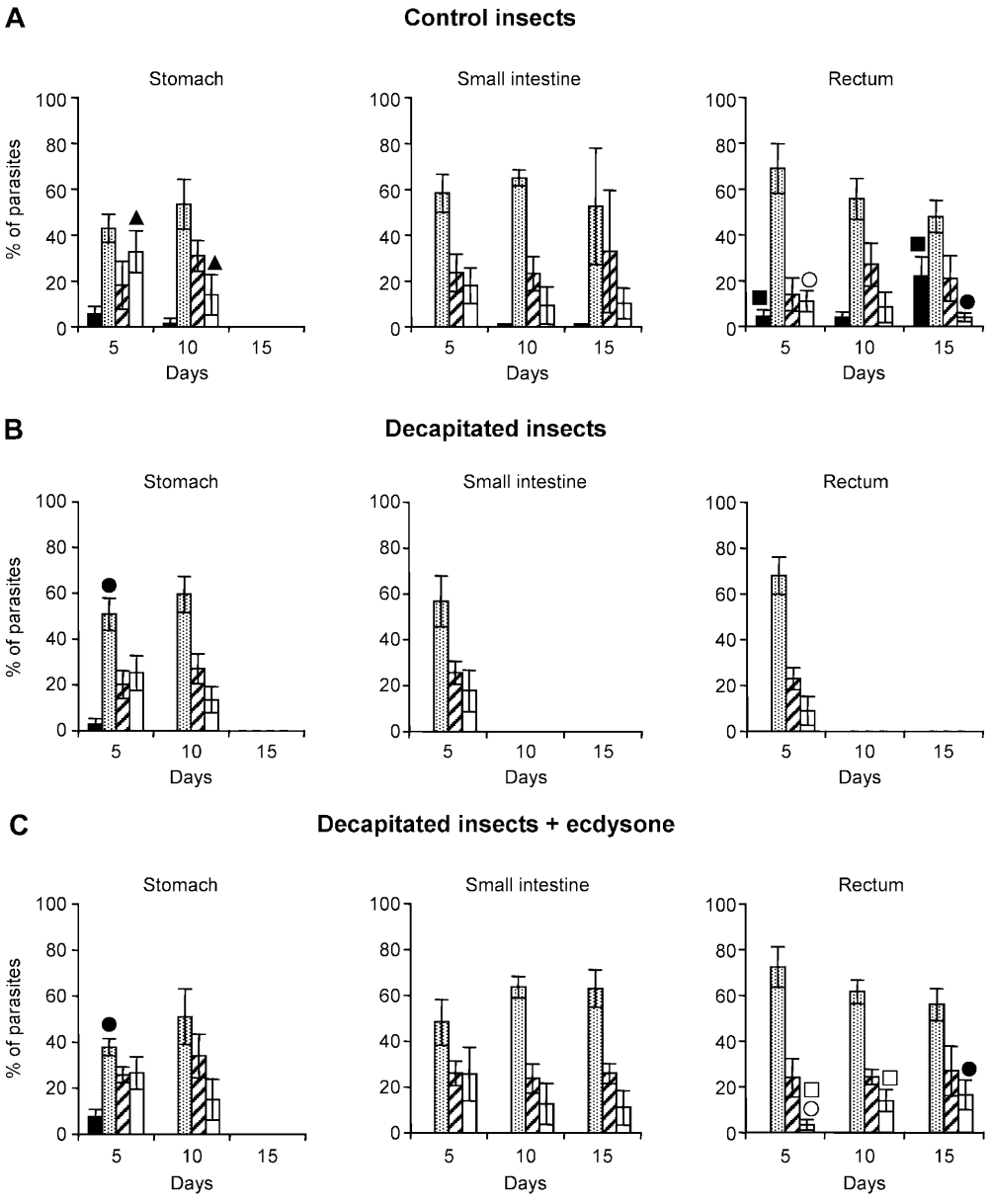

Fig. 3A–C Effects of decapitation and ecdysone therapy on developmental forms of *T. cruzi* Dm28c clone in different gut compartments of fifth-instar larvae of *R. prolixus*. Insects were fed on blood containing 3.0×10^6 parasites/ml and then decapitated 1 day after feeding. Ecdysone-treated insects received a single dose of α-ecdysone in the blood meal (5 μg/ml). **A** Control insect, **B** decapitated insect and **C** ecdysone-treated decapitated insect. *White column* round forms, *speckled column* epimastigotes, *hatched column* intermediary forms between epimastigotes (or spheromastigotes) and trypomastigotes, *black column* metacyclic trypomastigotes. Each histogram represents the average ± SD of different forms of parasites in each gut compartment ($n=6$ insects). *White circle above column* $P<0.01$, *all other symbols above column* $P<0.005$

compared to controls at 5 and 10 days p.i. (Fig. 3A, C; $P<0.01$ for 5 days p.i., $P<0.005$ for 15 days p.i.).

No intermediate forms between epimastigotes and round forms were found in any gut compartment (stomach, small intestine, rectum) during the entire experimental period.

Investigating the population density and the percentages of the metacyclic trypomastigote at 30 days p.i. (Table 1) in the whole gut of *R. prolixus*, the total number of parasites in the control insects was similar to that found in the small intestine plus rectum at 15 days p.i. (Table 1). No parasites were detected in the group of decapitated insects at this dissection date. Again, the total number of parasites in the gut was similar in the control and those insects decapitated but fed with blood supplemented with ecdysone, possessing $3.4\pm1.8\times10^5$ and $3.1\pm1.8\times10^5$ parasites/bug, respectively. At day 30 p.i., similar to the data from the rectum at 15 days p.i., 23% of the parasites in the control group were metacyclic trypomastigotes, but ecdysone-treated decapitated insects contained 8% of metacyclic trypomastigotes in the total population (Table 1; $P<0.001$).

Discussion

Trypanosoma cruzi undergoes many distinct biochemical and morphological changes during the life cycle, for survival within the gut of the insect vector, for invasion of and survival within mammalian cells and for evading the immune system of the mammalian host. If the vector ingests infected blood, the blood trypomastigotes not only transform mainly into epimastigotes in the lumen

Table 1 Development and metacyclogenesis of the *Trypanosoma cruzi* Dm 28c clone in the whole digestive tract of fifth-instar larvae of *Rhodnius prolixus* at day 30 post-infection. Insects were fed on blood containing 3.0×10^6 parasites/ml and then decapitated 1 day after feeding. Ecdysone-treated insects received a single dose of α-ecdysone in the blood meal (5 μg/ml). Each point represents mean ±SD of the parasites in six insects. – No data

Treatment	Total number of flagellates ($\times10^5$)	Total number of trypomastigotes ($\times10^5$)	Percentage of trypomastigotes
Controls	3.4 ±1.8	0.8 ±0.6	23.0*
Decapitated insects	0	0	–
Decapitated insect + ecdysone	3.1 ±1.8	0.2 ±0.2	8.0*

of the digestive tract, but also into spheromastigotes and both forms multiply and colonize the whole intestinal tract. Later, in the rectum, they transform into metacyclic trypomastigotes, non-dividing invasive forms that are transmitted to the mammalian host in the insect's feces or urine by contaminative infection. In the mammalian host, *T. cruzi* invades macrophages and muscle cells and multiplies as an amastigote, which afterwards differentiates into trypomastigotes, subsequently released into the blood to infect other cells or the invertebrate vector (for reviews, see Brener 1973; Garcia and Azambuja 1991; Azambuja and Garcia 1997; Kollien and Schaub 2000).

The density and stage composition of the parasite population in the bug is determined and affected by the different regions of the intestine and the change of the conditions there, induced by starvation or feeding of the vector (Kollien and Schaub 1998, 2000). In the *T. cruzi*–triatomine interaction model, metacyclogenesis predominates in the rectum (Dias 1934; Brack 1968; Zeledón 1987; Schaub 1989; Perlogowara-Szumlevicz and Carvalho-Moreira 1995). Thus, it is possible that some biochemical factors accumulated in the rectum, such as peptides derived from hemoglobin (Frandeirach et al. 1993; Garcia et al. 1995) or some urine components (Schaub and Lösch 1988; Zeledón et al. 1988; Montoreano et al. 1990; Kollien and Schaub 1998; Kleffman et al. 1998; Cabral et al. 2001), are involved in the parasite cycle in the vector.

The general pattern of infection by the *T. cruzi* Dm28c clone in the whole gut of *Rhodnius prolixus* investigated previously (Garcia et al. 1989a, b; Gonzalez and Garcia 1992; Gonzalez et al. 1999) was similar to the population development in the present investigation. Here, we demonstrate that, in the stomach, the parasite population decreased in all groups. In the small intestine and the rectum of decapitated bugs, the parasites established themselves for only a short period of time; and at 10 and 15 days p.i. parasites could not be detected there. The effect of decapitation was partially counteracted by feeding with ecdysone-supplemented blood 24 h prior to the decapitation.

Considering not only the parasite density but also the composition of the population, the present investigation can offer no new data about decapitated insects. However, it clearly shows that two forms of *T. cruzi* are affected by the decapitation of larvae supplemented with ecdysone: the round forms and the metacyclic trypomastigotes (including their intermediate stages). Round forms, nearly exclusively spheromastigotes, were first mentioned developing after infection of the bugs (Brack 1968). In the present investigation, this is also evident in all populations, their percentages decreasing to low levels (<10%) with the increasing period of infection. However, in ecdysone-treated decapitated bugs, the percentages increased up to 15 days p.i. in the rectum. This effect may be due to the second induction factor, stress conditions like starvation, as emphasized by Kollien and Schaub (1998, 2000).

In the control group and ecdysone-treated decapitated insects, the intermediate stages to metacyclic trypomastigotes showed no significant differences in both the small intestine and the rectum, during the entire experimental period. Discussing these stages, we should also mention a difficulty in the classification of the stages of Schaub (1989). He classified the unrolling form as an intermediate stage between spheromastigotes and epimastigotes. However, light micrographs demonstrate that they might also be intermediates between spheromastigotes and trypomastigotes.

More important in the present investigation is the development of metacyclic trypomastigotes. Those appearing in the stomach seem to be due to a development from intermediate stages present in the mixture of in-vitro-culture-derived epimastigotes used for the infection. They presumably do not indicate that metacyclic trypomastigotes usually develop in the stomach. However, the present data clearly demonstrate the importance of the rectum for the development of this stage. Only very low percentages developed in the small intestine. This is identical to previous investigations (see above). However, although decapitation with a previous ecdysone supplementation was sufficient to allow the development of high numbers of parasites in all gut regions, it was not sufficient to allow the development of metacyclic trypomastigotes within 15 days p.i. Even at 30 days p.i., only a significantly lower percentage of this form had developed. The partial reversion of the decapitation effect on the population density and on the development of metacyclic trypomastigotes by ecdysone therapy demonstrated that this hormone is able to modulate the differential balance between morphological stages of *T. cruzi* in the invertebrate host.

It is well known that decapitation of *R. prolixus* before HCP blocks the release of PTTH and consequently reduces the secretion of ecdysone from the prothoracic

glands, blocking the molting process (Wigglesworth 1934a; Garcia et al. 1990). Furthermore, ecdysteroids were described in the gut of triatomines (Steel et al. 1982; Stoka and Noriega 1982).

The changes in hormone concentrations seem to directly affect the gut. Insect decapitation before HCP induces significant changes in the epithelial cell organization of the gut of *R. prolixus* (Gonzalez et al. 1998), which after a blood meal, is normally composed of epithelial cells and an associated membrane system (see Billingsley and Downe 1983, 1986, 1988; Terra 1990). These decapitation-induced modifications included a disorganization of both the extracellular membrane layers and the basal portion of the epithelial cells. Furthermore, Gonzalez et al. (1999) demonstrated in converse experiments, that head transplantation or therapy with ecdysone re-established the organization of the epithelial cells and associated membranes (Gonzalez et al. 1998, 1999). These results indicate that a brain factor, possibly PTTH, acts on both the gut-cell organization and the gut microenvironment, interfering with trypanosome survival and infection of the vector. Thus, it is reasonable to suppose that ecdysone acts directly or indirectly on the gut epithelial cells of the insect determining the production of the perimicrovillar membrane necessary for the development of epimastigotes, intermediate forms and round forms of flagellates, but not metacyclic trypomastigotes. The latter seem to be affected by the change of, so far, unknown factors in the rectum.

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