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Changes of nuclear phenotypes in *Panstrongylus megistus* (Hemiptera, Reduviidae) under different stress conditions

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Abstract — The effect of fasting and of fasting followed by refeeding and heat shock was studied in Malpighian tubules of fourth instar nymphs of the blood-sucking hemipteran, *Panstrongylus megistus* (Burmeister). The aim was to detect different frequencies of nuclear changes (apoptosis, necrosis, heterochromatin decondensation) under conditions assumed to be stressful in blood-sucking hemipterans. The insects were fasted for up to 90 days at 28ºC and their survival was followed daily. Groups of nymphs were separated each month, with part of the group being refed and the other part kept fasting. Insects in each of these subgroups received either a heat shock at 40ºC for 1 h or were maintained at 28ºC (control for heat shock). The Malpighian tubules were removed one and seven days after each assay and subjected to the Feulgen reaction for identification and counting of the various nuclear phenotypes. Insect survival was high (90%) even after 40 days of starvation but decreased thereafter. Necrosis rather than apoptosis, increased with fasting. Feeding after fasting increased the frequency of apoptosis but not of necrosis. The short heat shock as used here did not additionally affect the responses induced by fasting and refeeding. *P. megistus* nymphs could withstand relatively long periods of fasting although individual variation in the mean length of cell survival had been found especially after a three-month fasting. The results related to cell necrosis suggest that part of the Malpighian tubule cells may not have developed highly efficient mechanisms for dealing with fasting. For those cells resistant to fasting, feeding subsequent to fasting acted only as a mild stressing agent and heat shock was well tolerated. The ability of *P. megistus* nymphs to withstand and recover from periods of inadequate or poor nutrition inclusive in association to a short heat shock as demonstrated here is certainly an important adaptation for the survival of the species.

Key words: fasting, heat shock, nuclear phenotypes, *Panstrongylus megistus*, refeeding

INTRODUCTION

Panstrongylus megistus, a blood-sucking hemipteran, is one of the most important vectors of Chagas' disease, because of its wide geographical distribution, high rates of infection with *Trypanosoma cruzi* and varied domiciliary behaviour (Forattini 1980). *P. megistus* is a native Brazilian species the domiciliation of which has increased markedly as a result of habitat destruction which has resulted in the elimination or reduction of natural food resources (Silveira 2000). Chagas' disease affects 20 million people, mostly in Central and South America, and is the third largest cause of death by infectoparasitary diseases in Brazil (Silveira and Rezende 1994; Dos Reis 1997). Although a decrease in the rural human population favors a reduction in the vectorial transmission of Chagas' disease in Brazil, this route of transmission is still the most frequent one, thus reinforcing the relevance of studies on the biology of blood-sucking hemipterans and their responses to several stressing agents, as part of control programs and for rearing these insects in the laboratory (Rodrigues *et al.* 1991; Silva and Silva 1993; Garcia *et al.* 1999; Schmunis 2000).

The 1st to 5th nymphal instars of blood-sucking hemipterans require blood meals every three to four weeks whereas adults need to feed every two weeks (Garcia and Azambuja 2000). These

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periods are sufficient for the insects to digest their meals. In the case of nymphs, the period indicated above is necessary for distension of the insect abdomen and stimulation of the prothoracic glands as well as for secretion of the molting hormone ecdysone (WIGGLESWORTH 1984). The frequency of feeding in these hemipterans depends on the temperature and relative humidity of the environment, as well as on the species considered, and the time required for each moult (GARCIA and AZAMBUJA 2000). In spite of ingesting an amount of blood many times greater than their body weight, blood-sucking hemipterans may also fast for long periods (SHERLOCK 1979). Resistance to starvation in blood-sucking hemipterans is thus of great epidemiological importance (BRAGA and LIMA 2001).

Changes in the nuclear phenotypes indicative of cell survival and cell death have been described in the Malpighian tubules of blood-sucking hemipterans subjected to some stressing agents, including fasting, heavy metals and heat shocks (MELLO and RAYMUNDO 1980; MELLO 1978 and 1989; MELLO *et al.* 1995 and 2001; GARCIA *et al.* 1999 and 2000). The normal nuclear phenotype in *P. megistus* shows one small heterochromatic body contributed by the Y chromosome (MELLO *et al.* 1986). Nuclei with heterochromatin decondensation have been considered to reflect an attempt to activate silent genes with stress (MELLO 1989). Nuclear phenotypes characteristic of apoptosis and necrosis (KERR 1971; KERR *et al.* 1972) have been defined in blood-sucking hemipterans in terms of their classic morphological characteristics (GARCIA *et al.* 2002; MELLO 2005). Cell and nuclear fusions may occur during fasting up to producing degrees of somatic ploidy higher than those normally reached through DNA endoreplication (MELLO 1978 and 1989; MELLO and RAYMUNDO 1980). Fasting may also result in an increase in the frequency of heterochromatin decondensation as a form of cell survival, as well as in the frequency of apoptosis and necrosis in the blood-sucking hemipteran *Triatoma infestans* (ANDRADE and MELLO 1987; MELLO 1989; MELLO *et al.* 2001).

Refeeding has been reported as a stressing factor in animal groups other than hemipterans when practiced after prolonged starvation (BURDON 1986). However, the survival of fasted specimens of *Triatoma infestans* has been shown to be unaltered by subsequent feeding (MELLO 1989). In the laboratory of Chagas' disease vectors at SUCEN (Mogi-Guaçu-SP), *P. megistus* specimens are often subjected to short periods of fasting in order to extend their developmental cycle (V.L.C.C. RODRIGUES, unpublished data). This routine procedure may thus provide a good model for investigating whether refeeding acts as an additional stressing factor in fasted blood-sucking hemipterans.

Nuclear phenotypes in *P. megistus* have been previously found to change with heat and cold shocks (GARCIA *et al.* 2000 and 2002). However, data in response to other stressing conditions like fasting, refeeding after fasting, and heat shock following fasting or refeeding after fasting are not as yet available. These data would be required as an attempt for the understanding of the species' survival under unfavorable environmental conditions. In the present study, the whole nuclear population of Malpighian tubules of *P. megistus* nymphs was investigated after the above-mentioned stressing conditions in terms of nuclear changes and quantification of each altered phenotype.

MATERIALS AND METHODS

Animals - Fourth instar nymphs of a domestic population of *Panstrongylus megistus* (Burmeister) reared at 28ºC and 80% relative humidity, short light regime, and fed with hen blood once a week in the laboratory at SUCEN (Mogi-Guaçu, SP) were used.

Experimental conditions - Four hundred nymphs were fasted for up to 90 days and monitored daily to score changes in their survival rate. Groups of nymphs were separated each month, with part of the group being refed also with hen blood, while the other part was kept fasting. Insects in each of these subgroups subsequently received a heat shock at 40ºC for 1 h before being returned immediately to the control temperature (28ºC) or were maintained at 28ºC all the time (control for heat shock). One and seven days later, the nymphs were dissected to remove their Malpighian tubules. As a general control group, insects fed with hen blood once a week were also examined.

Cell preparations - The Malpighian tubules were mounted *in toto* on glass slides, fixed in ethanol-acetic acid (3:1, v/v) for one min, rinsed in 70% ethanol for up to five min and then subjected to the Feulgen reaction with hydrolysis done for 65 min in 4 M HCl. They were subsequently rinsed with three washes in sulfurous water (five min each) and one wash in distilled water before being

air dried. The preparations were cleared in xylene and mounted in Canada balsam.

The total number of epithelial cell nuclei in the Feulgen-stained Malpighian tubules and the number of the different nuclear phenotypes under each experimental condition were counted. Two to four insects under each experimental and control condition were used. Photomicrographs were obtained using a Zeiss Axiophot 2 microscope (Oberkochen, Germany).

Statistics - Insect survival curves were estimated according to the KAPLAN-MEIER's (1958) procedure.The survival rates of fully-nourished and fasted insects were compared using the MANTEL-HANTZEL test (KALBFLEISH and PRENTICE 1980). The frequencies of nuclear phenotypes in Malpighian tubules under the various experimental conditions were compared using ANOVA after convenient stabilization and normalization of the data using an arc sin transformation (LITTLE and RUBIN 1987).

RESULTS

The survival rate in fasted insects was high (90%) after up to 40 days but decreased drastically thereafter. After 90 days of fasting, the survival rate of the nymphs was approximately 42% (Fig. 1). Comparison of the survival rates of fasted and well-nourished nymphs showed that there was a significant difference between the survival curves of these two groups, with a decreased survival in fasted specimens ($p = 8.35e\text{-}14$).

Normal nuclei predominated in nymphal Malpighian tubules under the various conditions (Fig. 2a, e). The phenotypic alterations observed consisted of nuclei with heterochromatin decondensation (Fig. 2b), apoptotic nuclei (Fig.2c, e), nuclei suspected of apoptosis (Fig. 2d), and necrotic nuclei (Fig. 2d). Giant nuclei were not observed. Nuclei with heterochromatin decondensation do not reveal the heterochromatic body as in normal nuclei (Figs. 2a, b). Apoptotic nuclei showed a deeply stained and densely packed nuclear mass; sometimes differently sized vesicles were seen budding from the main nuclear body (Fig. 2c). Nuclei suspected of apoptosis were those in which euchromatin (originally less packed chromatin) stained with the Feulgen reaction nearly as deep as the heterochromatic body but their size and shape are not yet those expected from classic apoptotic images (Fig. 2d). Necrotic nuclei

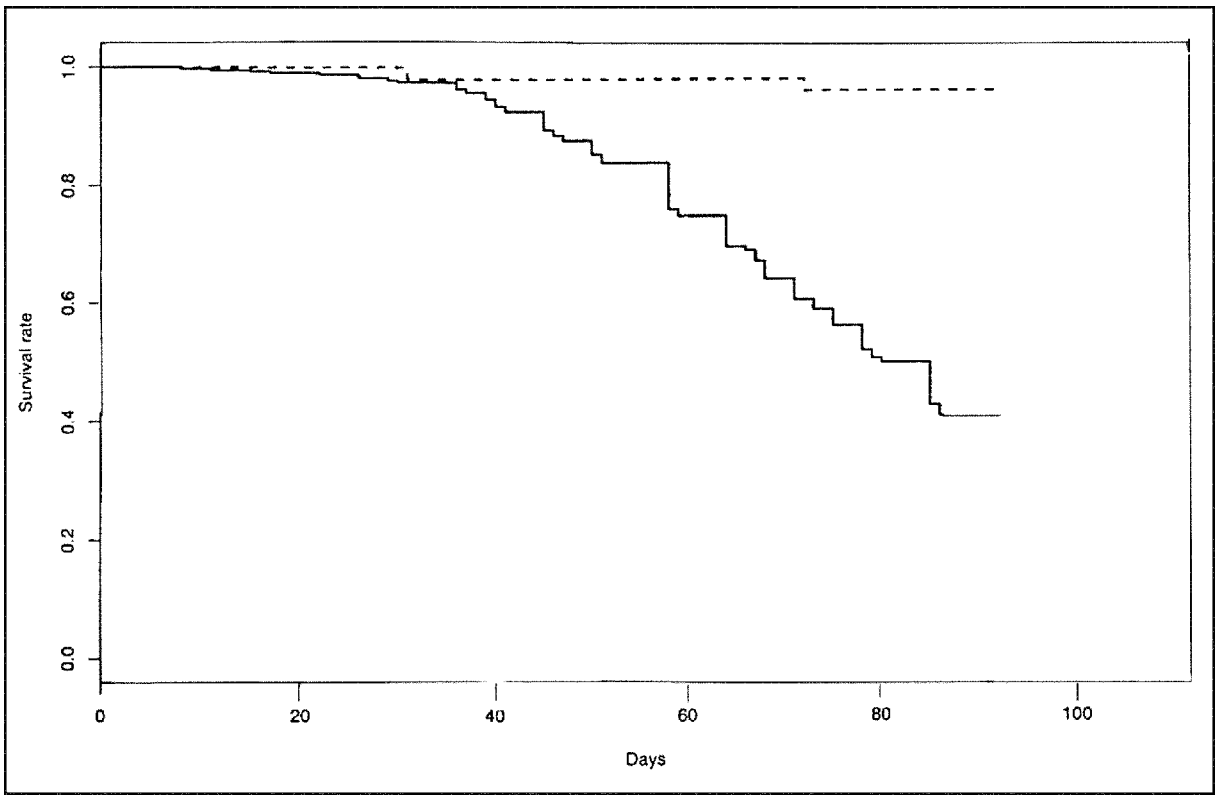

Fig. 1 — Survival curves of fully-nourished (dashed line) and fasted (full line) *P. megistus* nymphs.

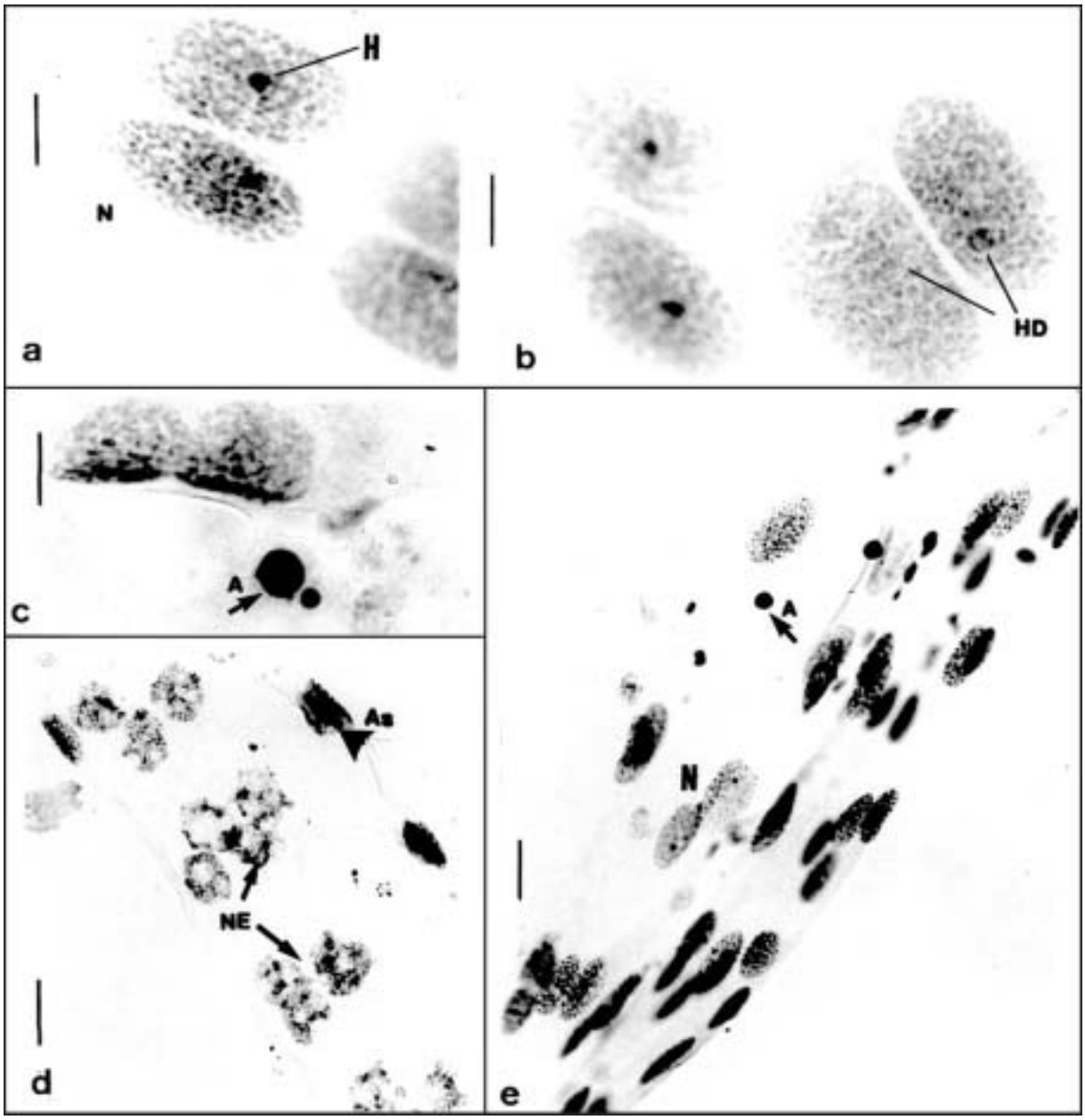

Fig. 2 — Nuclear phenotypes defined for Feulgen-stained *P. megistus* Malpighian tubules. a) normal nuclei (N) with one conspicuous heterochromatin body (H) (arrow); b) nuclei with heterochromatin decondensation (HD) (arrows); c) nuclear image of an apoptotic cell (A) (arrow); d) nuclear images of suspected apoptosis (As) (arrowhead) and necrosis (NE) (arrows); e) general view of normally nucleate (N) and of two apoptotic cells (A) (arrow). The bars indicate 10 μm (a-c) and 20 μm (d, e).

showed a Feulgen-stained chromatin framework that culminated in nuclear disruption (Fig. 2d).

The frequencies of the nuclear phenotypes counted in fasted and refed specimens with or without heat shock are shown in Tables 1 and 2. The effect of the conditions fasting time, refeeding, heat shock and time after heat shock considered separately on the various phenotypes was analysed statistically, as shown in Table 3.

Normal nuclei - The frequency of nuclei with a normal phenotype decreased significantly with increasing duration of fasting but was not affected by refeeding (Tables 1-3). Heat shock decreased the frequency of this phenotype, especially in specimens fasted for three months (Tables 1 and 3), but there was generally no association between heat shock and fasting (Tables 4 and 5).

Table 1 — Absolute frequency of nuclear phenotypes in Malpighian tubules of fasted *P. megistus* subjected to heat shock at 40ºC for 1 h.

Test Conditions	Time after				Nuclear phenotype arithmetic means				
	Fasting time (months)	Heat Shock (HP)	HP or corresp-onding control (days)	n	Apop-tosis	Nuclei suspected of apoptosis	Necrosis	Hetero-chromatin decon-densation	Normal nuclei
Experimental	1	Yes	1	3	0.0	4193.0*	1984.0	1598.3*	12348.7
		Yes	7	2	0.0	4690.0*	1728.5	849.0*	10802.0
	2	Yes	1	2	0.0	166.0	993.0	302.5*	9794.0
		Yes	7	3	0.0	151.3	990.7*	153.7	12529.0
	3	Yes	1	4	0.0	77.8	1088.8*	207.2*	8139.8
		Yes	7	2	0.0	15.0*	1385.5	350.5*	7856.0
Control	1	No	1	3	1.0*	2447.7*	1270.0	74.3*	16690.3
		No	7	3	0.3*	2321.3	621.7	383.3*	13254.0
	2	No	1	3	0.0	310.7	799.0	415.7*	13636.3
		No	7	3	0.0	99.7*	1284.3*	190.7	9902.3
	3	No	1	3	3.0*	49.3	921.3*	411.0*	10685.3
		No	7	2	2.0*	131.0	4978.0	116.0*	10587.0

*: standard deviations > half arithmetic means; n: number of insects

Table 2 — Absolute frequency of nuclear phenotypes in Malpighian tubules of fasted *P. megistus* refed and then subjected to heat shock at 40ºC for 1 h.

Test Conditions	Time after				Nuclear phenotype arithmetic means				
	Fasting time (months)	Heat Shock (HP)	HP or corresp-onding control (days)	n	Apop-tosis	Nuclei suspected of apoptosis	Necrosis	Hetero-chromatin decon-densation	Normal nuclei
Experimental	1	Yes	1	3	0.0	2693.0	1654.7	345.0*	13204.7
		Yes	7	3	2.3*	2268.0*	2154.3	250.0*	14834.7
	2	Yes	1	3	1.3*	245.0*	1088.7*	189.3*	10284.0
		Yes	7	2	0.0	230.0*	943.5*	447.0*	11032.5
	3	Yes	1	3	0.0	120.7*	3169.0	220.7*	10825.0
		Yes	7	3	1.0*	520.0	1751.3*	248.0*	8055.3
Control	1	No	1	3	2.3*	3270.3	1172.0	13.0*	14174.0
		No	7	3	9.0	2231.3*	1812.3*	373.0*	15951.7
	2	No	1	4	2.5*	315.2*	933.0	86.5*	9671.2
		No	7	3	0.3*	765.7*	740.7*	819.3*	11273.7
	3	No	1	3	2.3*	84.3*	1406.7*	69.3	8214.0
		No	7	3	1.7*	138.7*	1811.3*	149.0*	10003.7

*: standard deviations > half arithmetic means; n: number of insects

Table 3 — Analysis of variance (ANOVA) for nuclear phenotypes in *P. megistus* Malpighian tubules under the various experimental conditions considered in separate.

Nuclear phenotypes	Effects Duration of fasting	Refeeding	Heat shock	Time for cytological analysis after the last treatment
Apoptosis	0.967	<u>0.005</u>	<u>0.004</u>	0.539
Nuclei suspected of apoptosis	<u>0.000</u>	0.563	0.680	0.822
Necrosis	<u>0.000</u>	0.151	<u>0.035</u>	0.428
Heterochromatin decondensation	0.756	0.153	0.115	0.287
Normal nuclei	<u>0.035</u>	0.490	<u>0.050</u>	0.462

Significance at $p \leq 0.05$ is underlined.

Table 4 — Frequency of nuclear phenotypes in fully-nourished, fasted and refed *P. megistus* nymphs subjected to heat shock at 40ºC for 1 h.

Feeding conditions	n	Frequency arithmetic means				
		Apoptosis	Nuclei suspected of apoptosis	Necrosis	Hetero-chromatin decondensation	Normal nuclei
Fully-nourished						
Unshocked control	13	0.8*	142.8*	797.5*	31.5	10669.2
Shocked	12	3.3*	115.7*	541.5*	70.5*	10827.0
Fasted						
Unshocked control	17	1.0*	938.1*	1547.5*	273.9*	12569.3
Shocked	16	0.0	1442.9*	1382.8*	568.0*	10255.8
Fasted + refed						
Unshocked control	19	3.0*	1091.1*	1467.6*	243.0*	11449.3
Shocked	17	0.8*	1058.8*	2061.6	273.7*	11392.7

*: standard deviations > half arithmetic means; n: number of insects

Table 5 — Analysis of variance (ANOVA) for comparison of frequencies of nuclear phenotypes under full nourishment, fasting, and fasting + refeeding nutritional conditions (N), and under heat shock associated with these conditions (N + HS).

Nuclear phenotypes	Effects	
	N	N + HS
Apoptosis	0.204	0.116
Nuclei suspected of apoptosis	<u>0.012</u>	0.802
Necrosis	<u>0.017</u>	0.284
Heterochromatin decondensation	<u>0.000</u>	0.051
Normal nuclei	<u>0.000</u>	0.199

Significance at $p \leq 0.05$ is underlined.

Heterochromatin decondensation - This phenotype was not affected with increasing duration of fasting (Tables 1 and 3). Only in insects fasted for one month did heat shock increase the frequency of heterochromatin decondensation (Table 1). No significant differences were observed after a general analysis (Table 3). On the other hand, refeeding decreased the frequency of heterochromatin decondensation mainly in non-shocked insects one day after a one month fast in comparison with fasted-only specimens (Tables 1 and 2).

Apoptosis - Apoptosis was not elicited by fasting or by heat shock at 40ºC for 1 h (Table 1). However, the refeeding condition significantly induced apoptosis, especially after fasting for one month (Tables 2 and 3). Heat shock given to the refed specimens decreased the frequency of apoptosis, especially shortly after its application (Table 2).

Nuclei suspected of apoptosis - The frequency of this phenotype decreased significantly with the duration of fasting (Tables 1 and 3), but was not additionally affected by heat shock in fasted specimens. Heat shock increased suspected apoptosis only after a one-month fast. Refeeding either increased the frequency of suspected apoptosis shortly after a one month fast or reduced it in refed specimens subjected to heat shock (Tables 1 and 2). Refeeding with or without subsequent heat shock had a variable effect on the frequency of nuclei suspected of apoptosis in nymphs fasted for two and three months (Table 2). In specimens refed after the three month fast, the frequency of nuclei suspected of apoptosis increased seven days after the heat shock (Table 2). The unpredictable change in the frequency of this phenotype according to the stress conditions (Table 3) meant the effect of refeeding was not significant in establishing cause-effect relationships.

Necrosis - Necrosis was significantly affected with increasing duration of fasting (Tables 1 and 3). Refeeding with or without subsequent heat shock increased the frequency of necrosis only in specimens fasted for three months (Tables 2 and 3). However, the effect of refeeding in general terms was not significant for changes in frequency of the nuclear phenotype characteristic of necrosis (Table 3). Although heat shock isolatedly increased

the frequency of necrosis in the Malpighian tubules of *P. megistus* (Table 3), it did not significantly affect the effect promoted by fasting plus refeeding (Table 5).

There was a significant difference in the frequency of nuclei suspected of apoptosis, of necrotic nuclei, of nuclei with heterochromatin decondensation, and of normal nuclei in fasted or fasted then refed specimens compared to the well-fed insects (Tables 4 and 5).

DISCUSSION

The fact that nearly 50% of the nymphs were alive 80 days after fasting agreed with reports showing that blood-sucking hemipterans can withstand long periods of fasting in the laboratory (Sherlock 1979; Mello and Raymundo 1980; Mello 1989). Resistance to starvation is of great epidemiological importance because under unfavorable conditions involving temperature extremes and desiccation the insects may seek refuge in holes in house walls where they can remain for relatively long fasting periods, thus escaping from the effects of the harmful agents and increasing the blood-sucking hemipteran's opportunities for reproduction and resettlement (Braga and Lima 2001).

The large standard deviations observed for the frequencies of many of the nuclear phenotypes studied here indicated considerable individual variations in the mean length of cell survival especially after a three-month fasting. However, comparison of the data acquired confidence since ANOVA was carried out only after the data were conveniently stabilized and normalized through an arc sin transformation (Little and Rubin 1987).

The finding that only the frequency of necrotic nuclei increased with advancing fasting time in *P. megistus* nymphs suggests that part of the cells of this species may be relatively uncapable to develop efficient mechanisms of survival (heterochromatin decondensation, for instance) such as those seen in *Triatoma infestans* under similar physiological conditions (Mello 1989; Mello *et al.* 2001). Heterochromatin decondensation has been detected in several organisms under various stress conditions, among which heat shocks and heavy metals (Simoes *et al.* 1975; Mello 1989; Mello *et al.* 1995; Michailova *et al.* 1996; 2001; Rizzi *et al.* 2004), and considered to represent an attempt or a successful transcriptional activation of silent genes in response to unfavorable conditions of stress stimuli (Mello 1989; Rizzi *et al.* 2004).

The absence of giant nuclei in the Malpighian tubules of fourth instar nymphs of *P. megistus* subjected to a three-month starvation differed from a previous report showing that this nuclear phenotype appears after a four- or five-month starvation in adults and in late fifth instar nymphs of this species (Mello and Raymundo 1980). The decrease in the frequency of normal nuclei with increasing fasting is a function of nuclear elimination by cell death during fasting.

In the well-fed specimens of *P. megistus* analyzed here the number of nuclei counted per nymph was sometimes lower than that of nuclei from one-month fasted specimens. This was possibly due to some occasional tissue loss from the well-fed nymphs by the operator because of the high concentration of fat body cells and distension of the midgut in these specimens. Some unknown stressing effect during rearing of the insects in the laboratory is neither to be neglected. However, this does not invalidate the other differences in nuclear phenotypes found with advancing fasting and with refeeding.

The increase in the frequency of apoptosis in insects refed after fasting indicated that feeding after fasting acted as a stressing factor as previously reported for another cell system (Hervant *et al.* 2001), and that no protective mechanism, at least in terms of heterochromatin decondensation (Mello 1989; Mello *et al.* 2001), was elicited simultaneously. However, the stress induced by refeeding was not severe enough to increase the frequency of necrosis..

Heat shock under temperature and time conditions used here did not act as a stressing factor in addition to fasting and refeeding since it did not significantly affect the frequency of the various nuclear phenotypes observed. This finding agreed with the resistance of fasted specimens of *T. infestans* to combined stress factors such as fasting and heavy metals, and enhanced tolerance to sequential cold shocks (Mello *et al.* 1995; Campos *et al.* 2002). Probably heat-shock proteins preliminary activated by fasting or fasting plus refeeding conditions induced tolerance to a sequential stressor like a short heat shock (Welch 1993; Garcia *et al.* 2003), similarly to the effects promoted in *P. megistus* by sequential heat or cold shocks (Garcia *et al.* 2002; 2003).

P. megistus is the principal vector of *T. cruzi* in the eastern, southern and some northeastern states of Brazil (Barbosa *et al.* 2001). The ability of *P. megistus* specimens to withstand and recover

from periods of inadequate or poor nutrition inclusive in association to a short heat shock as presently shown is certainly an important adaptation for the survival of the species. However, the survival adaptation of *P. megistus* specimens to infection with *Trypanosoma cruzi* still deserves investigation. Studies on specimen survival, molting rate, and metacyclogenesis incidence in *T. cruzi*-infected *P. megistus* and on its cell survival and cell death responses are in progress.

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