

## NUCLEAR FUSION AND CHANGE IN CHROMATIN PACKING STATE IN RESPONSE TO STARVATION IN *Triatoma infestans*

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### ABSTRACT

Feulgen-stained nuclei of Malpighian tubule epithelial cells of *Triatoma infestans* 5th instar nymphs under conditions of full nourishment, prolonged starvation, and feeding following starvation ("recovery") were studied with scanning microspectrophotometry and image analysis. The aim was to correlate changes in nuclear phenotypes with any change in DNA content and/or chromocenter structure in response to the fasting stress. In the starved and "recovered" specimens, nuclei resembling those of the control as well as fused and degenerated nuclei were found. Nuclear fusion giving rise to ploidy degrees up to the 1024 C class were detected in nuclei of the starved insects, whereas very giant fused nuclei were absent in the "recovered" specimens, since they possibly degenerate. Changes in nuclear phenotypes involving heterochromatin unravelling could be demonstrated with image analysis for part of the fused and unfused nuclear populations of the starved and "recovered" specimens. The relatively unpacked state of the heterochromatin did not revert on cessation of the stressor action. Although starvation appears to be a specific stress factor capable of inducing nuclear fusion in *T. infestans* cells *in vivo*, the phenomenon of heterochromatin unravelling is possibly an attempt to activate (a) dormant gene(s), and should not be considered a fasting-specific response.

### INTRODUCTION

Nuclear fusion has been reported in specimens of several insect species of the family Reduviidae subjected to starvation (Wigglesworth, 1967; Mello and Raymundo, 1980; Mello, 1983; Andrade and Mello, 1987). This phenomenon has been observed to affect even cells in which the optimal ploidy degrees (32C and 64C classes) had been attained previous to starvation. In 3-month-starved 5th instar nymphs of

*Triatoma infestans* for instance, fusion occurs in up to 40% of the epithelial cell nuclei of the Malpighian tubules (Andrade and Mello, 1987).

The Malpighian tubules of 5th instar nymphs of *T. infestans* exhibit cell nuclei with one or with several chromocenters (Mello, 1971), which have been demonstrated to contain a constitutive heterochromatin (Mello and Recco-Pimentel, 1987). It is suspected that with starvation some of these chromocenters enlarge, surpassing the area expected from data for fully-nourished specimens or even if one considers the possibility of chromocenter fusion (fused nuclei). However, change in DNA content or in the chromatin packing state has not yet been demonstrated for these chromocenters under fasting conditions.

The purpose of this investigation was to study with scanning microspectrophotometry and image analysis Feulgen-stained Malpighian tubule nuclei in *T. infestans* nymphs subjected to prolonged starvation, in order to establish changes in nuclear phenotypes and correlate these with any change in DNA content and/or in supra-organization of the chromocenter structure associated with the fasting stress.

## MATERIALS AND METHODS

Malpighian tubules of 6.5-month starved 5th instar nymphs of *Triatoma infestans* Klug (Hemiptera, Reduviidae) were used. Fully-nourished specimens were employed as controls. The specimens under fasting were kept isolated in order to avoid cannibalism (Brumpt, 1914). Some starved specimens were analyzed 6 days after feeding on a blood meal ("recovery" assay). Organs were removed from at least three specimens for each experimental condition.

The tubules were fixed in acetic ethanol for 3 min and very gently pressed in a drop of 45% acetic acid onto slides. Care was taken not to disrupt the nuclei. Coverslips were removed with the dry ice technique. The slides were then immersed in 70% ethanol for 5 min and air dried. The material was subjected to the Feulgen reaction (basic fuchsin from Carlo Erba), hydrolysis being accomplished in 4M HCl at 22°C for 1 hr and 20 min. The preparations were mounted in Cargille oil ( $n_D = 1.55$ ).

### *Scanning microspectrophotometry*

Feulgen-DNA values in arbitrary units were obtained with a Zeiss automatic scanning microspectrophotometer linked to a Microdata computer. Operating conditions were: Planapo 100/1.25 objective, optovar 2, measuring diaphragm dia. = 0.1 mm, field diaphragm dia. = 0.2 mm, Zeiss LD-Epiplan 16/0.30 condenser, 0.5  $\mu\text{m}$  x 0.5  $\mu\text{m}$  scanning spot size and  $\lambda = 565$  nm obtained with a Schott monochromator filter ruler. The half band width at the spot transmission was quite small since the width of the effective light beam was equal to 0.2 mm (Zeiss information, 1977). A

predominantly monodirectional scanning motion was used. The grid points showing absorbances  $\leq 0.020$  were considered background and were automatically removed from the nuclear image. The nuclear and chromocentral Feulgen-DNA values and absorbing areas were determined for nuclei from the three treatment groups (control, starvation, "recovery").

### Image analysis

The descriptors used were:

1. Total integrated absorbance ( $Abs_T$ ): nuclear Feulgen-DNA values;
2. Area in  $\mu m^2$  covered by stained chromatin ( $S_T$ );
3. Nuclear average absorbance ( $\bar{A} = Abs_T/S_T$ );
4. Integrated absorbance over a pre-selected absorbance value ("cut off" point, c.o.) ( $Abs_C$ , chromocentral Feulgen-DNA values). In the present case, c.o. = 1.000, a value which was chosen after a preliminary test and was considered to discriminate absorbances of the chromocenters in the control;
5. Chromocentral average absorbance ( $\bar{A}_C = Abs_C/S_C$ );
6. Area in  $\mu m^2$  covered by the stained chromocenters ( $S_C$ );
7. Area in % covered by the chromocenters (chromocentral area) ( $S_C \% = (S_C/S_T) \times 100$ );
8. Average absorption ratio (AAR), which is the ratio of the chromocentral average absorbance to the whole nuclear average absorbance ( $AAR = \bar{A}_C/\bar{A}$ ) (Vidal *et al.*, 1983).

The Feulgen-DNA values were plotted as frequency histograms distributed on a scale of values in geometric progression (Bucher and Horisberger, 1950; Palkóvits and Fischer, 1968).

## RESULTS

The Malpighian tubules of the fasted specimens of *T. infestans* exhibited nuclei with the phenotypes described for fully-nourished insects (single- and multi-chromocentered nuclei) (Figures 1, 2, 4, 6). However, nuclei of the usual size, but containing partly unpacked chromocenters (Figure 3), and giant nuclei with irregular contours and a large and not very densely packed chromocenter (Figure 4) or with many small and deeply stained chromocenters (Figures 5 and 6) were also detected. Visual suggestions of steps preceding the nuclear fusion which will give rise to giant nuclei as well as the degenerated nuclear types previously described for specimens of *T. infestans* subjected to a much shorter fasting period (Andrade and Mello, 1987),

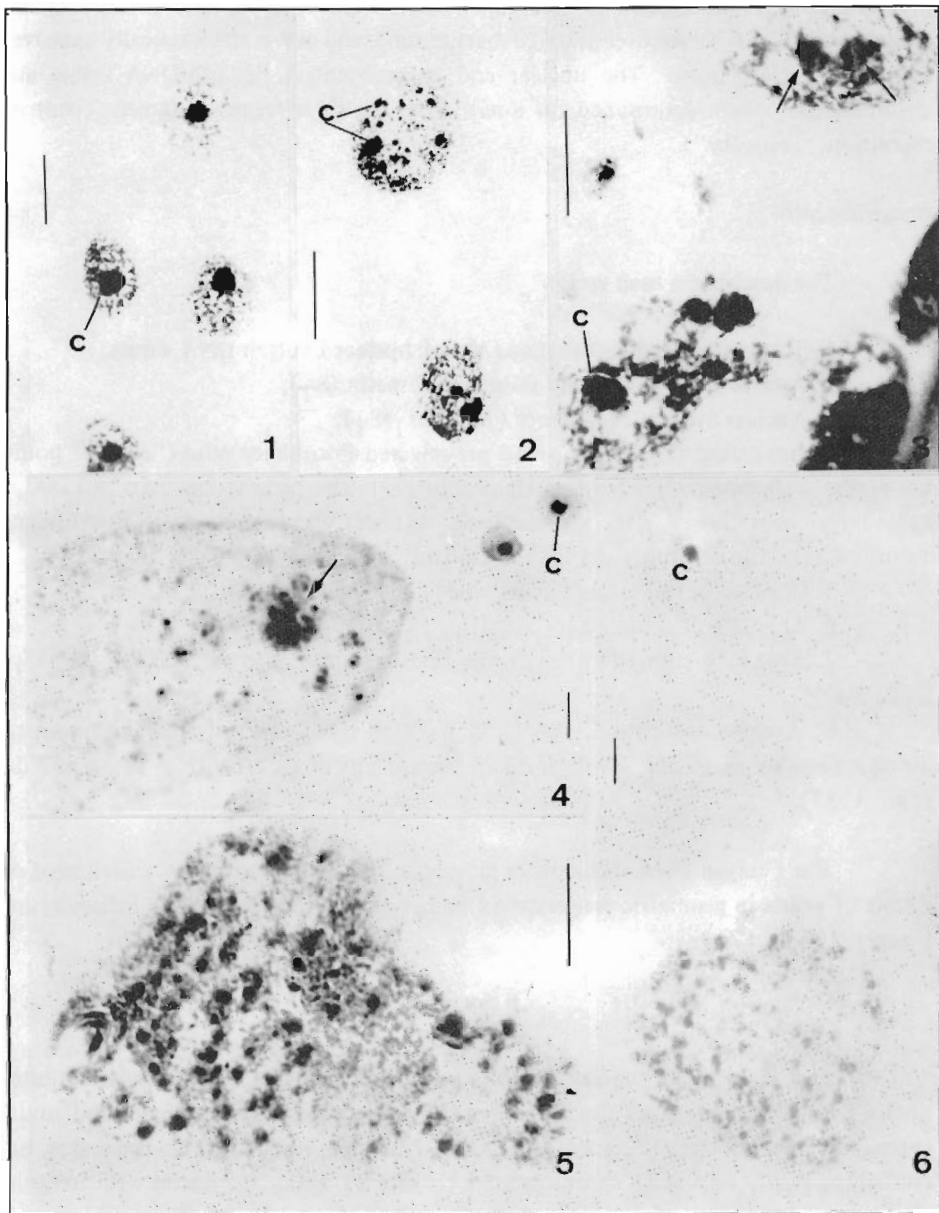


Figure 1 to 6 - Feulgen-stained single- and multichromocentered nuclei in Malpighian tubules of *T. infestans*. 1 and 2. Fully-nourished controls. 3 to 6. Starved specimens, giant nuclei being observed in Figures 4 to 6. C = chromocenters. Partly unpacked chromocenters are indicated (arrows). The bars equal 20  $\mu\text{m}$ .

were also observed. The same nuclear phenotypes appeared in the "recovery" assay, except that giant nuclei were much less frequent and exhibited signs of degeneration.

The Feulgen-DNA determination demonstrated values up to a 1024 C content for the fused nuclei of the fasted insects (Figure 7); considering that 5th instar tubules in fully-nourished *T. infestans* contain 32 C and 64 C nuclei, maximal values arose in these organs by DNA endoreplication (Mello, 1971, 1978). Fusion of nuclei with intermediate DNA content, when considering the doubling series, may account for values shifted to the extremity position in a doubling interval, as appears to occur for the distribution of values within the 128 C class (Figure 7). On the other hand, the frequency histograms of the Feulgen-DNA data for the "recovery" experiment, showed values up to the 128 C class only, which was to be expected from the absence of very giant nuclei.

As regards the Feulgen-DNA values of the chromocenters of the starved specimens, fused nuclei were found which exhibited values exceeding those found in the control, but other nuclei showed values equal to or notably smaller than those of the control (Figure 8). Single- and multichromocentered unfused nuclei of the fasted specimens and fused nuclei of the "recovered" specimens exhibited values lower than those of the control. A certain decrease in Feulgen-DNA values for the chromocenter of the single- chromocentered unfused nuclear population of the "recovered" insects was also observed (Figure 8).

The fact that the Feulgen-DNA content of the chromocenters does not always follow that of the whole nuclei in fused and in certain unfused nuclei of the starved and "recovered" insects can be visualized in Figure 9.

The chromocenter absorbing areas in multichromocentered fused nuclei of the fasted specimens, also calculated for the nuclear zones displaying absorbances  $> 1.000$ , were found to be larger than or equal to those of the control (Figure 10). Single- and multichromocentered unfused nuclei of the fasted insects, on the other hand, exhibited chromocentral absorbing areas equal to those of the 32 C control nuclei, whereas those nuclei of the "recovery" experiment exhibited chromocentral areas not exceeding those of the control (Figure 10).

When plotting AAR values against chromocentral areas in percentages, the distribution of the single- and multichromocentered fused nuclei of the starved insects appeared well discriminated from that of the control (Figures 11 and 12). The distribution of some of the unfused nuclei of the starved specimens also shifted from the curve established for the controls (Figure 11 and 12).

In controls, the chromocentral absorbing area of most nuclei was found in the 8.0 - 36.0% range, a distribution never observed in fasted and "recovered" insects. AAR values, in addition, were mostly lower than 4.5 (Figure 11). In the fasted insects, fused nuclei exhibited chromocentral absorbing areas smaller than 7.0% and AAR between values 3.0 and 6.0. Unfused nuclei had a chromocentral area no larger than

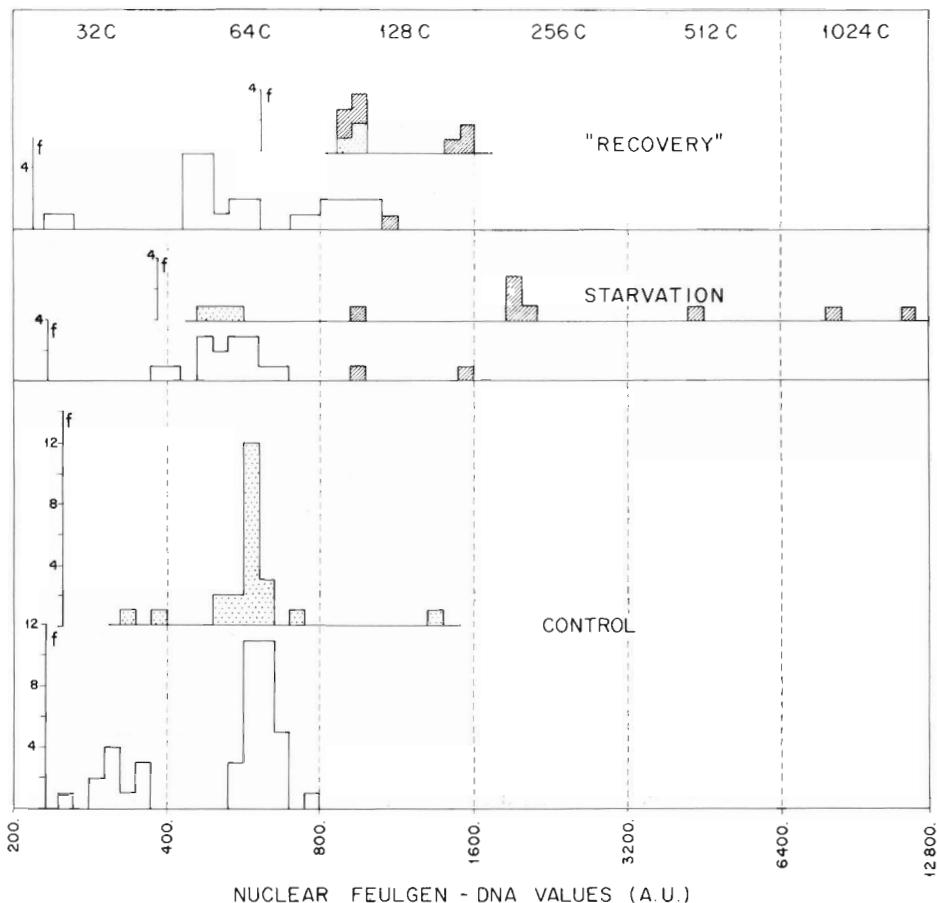


Figure 7 - Nuclear Feulgen-DNA content in arbitrary units (A.U.) for Malpighian tubule epithelial cells of *T. infestans*.  $f$  = frequency; open columns, single-chromocentered nuclei; dot-filled columns, multichromocentered nuclei; slant shaded blocks, fused nuclei.

2% and their AAR data were distributed between the values 6.0 and 10.0 (Figure 12). In the "recovered" insects, fused nuclei exhibited chromocentral areas lower than 5% and AAR was positioned between the values 4.5 and 8.0. The chromocentral areas of unfused nuclei were mostly smaller than 6% and AAR generally fell between the values 4.0 and 11.5 (Figure 12).

## DISCUSSION

The results indicate nuclear fusion and nuclear degeneration in part of the

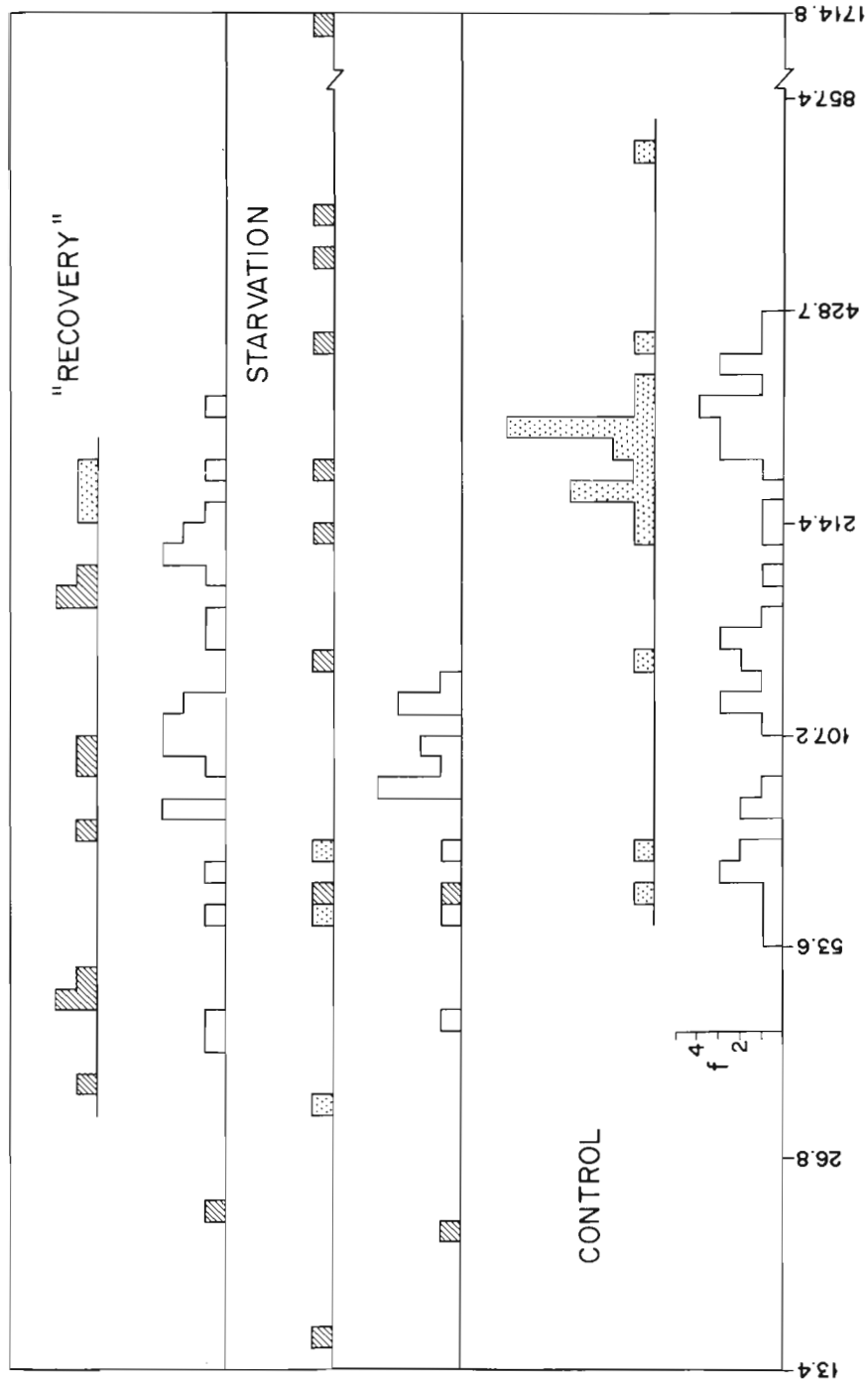


Figure 8 - Feulgen-DNA values in arbitrary units (A.U.) for chromocenters of *T. infestans*. f = frequency; open columns, single-chromocentered nuclei; dot-filled columns, multi-chromocentered nuclei; shaded blocks, fused nuclei.

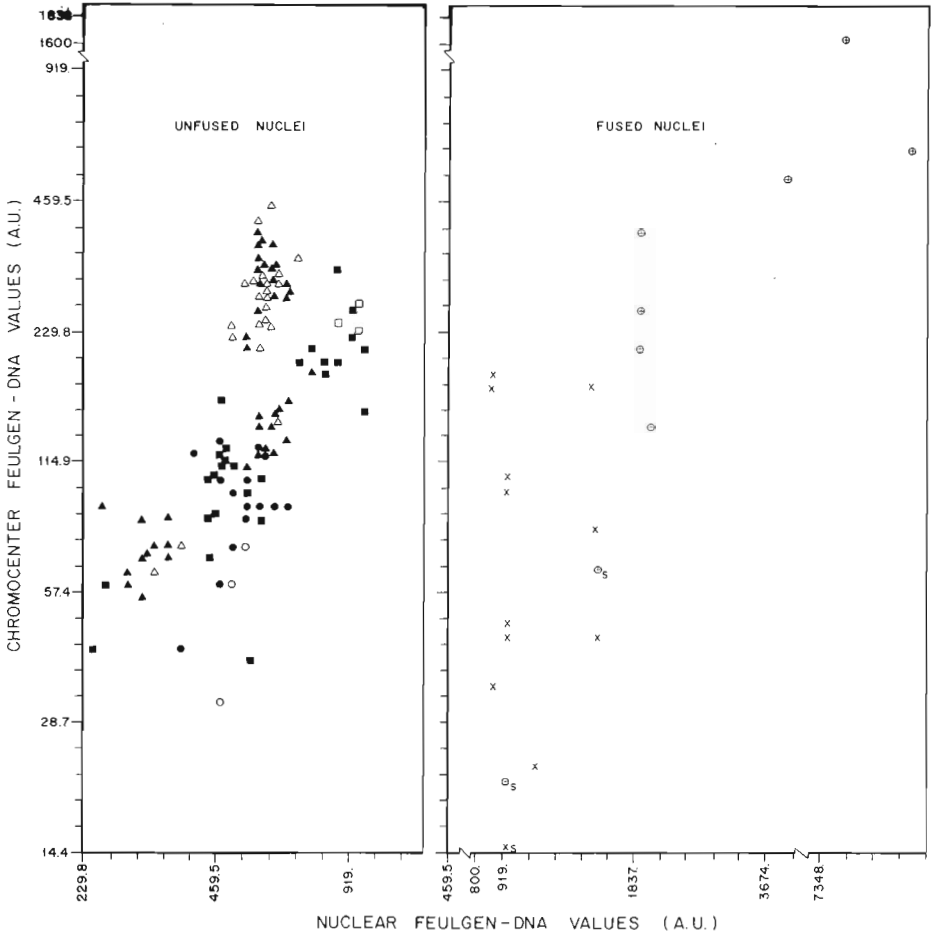


Figure 9 - Correlation of whole nuclear and chromocenter Feulgen-DNA values. A.U. = arbitrary units;  $\triangle$   $\blacktriangle$  = fully-nourished specimens;  $\circ$   $\oplus$   $\ominus$  = starved specimens;  $\square$   $\blacksquare$   $\times$  = "recovered" insects; empty symbols and S = single-chromocentered nuclei; filled symbols = multichromocentered nuclei.

cellular population of the Malpighian tubules of *T. infestans* subjected to severe starvation. This persisted even after a period of feeding following starvation, though very giant nuclei were then absent, probably because they develop an inability to survive in conditions of re-establishment of organ metabolism.

Nuclear fusion *in vivo* has been previously proposed for *T. infestans* specimens subjected to a much gentler fasting (Andrade and Mello, 1987), based not on Feulgen-DNA values, but on the 40% reduction of the total number of Malpighian tubule nuclei in the fasted insects. The evaluation of Feulgen-DNA values as carried out in this work, allows us to demonstrate that in addition to nuclear fusion, cell fusion

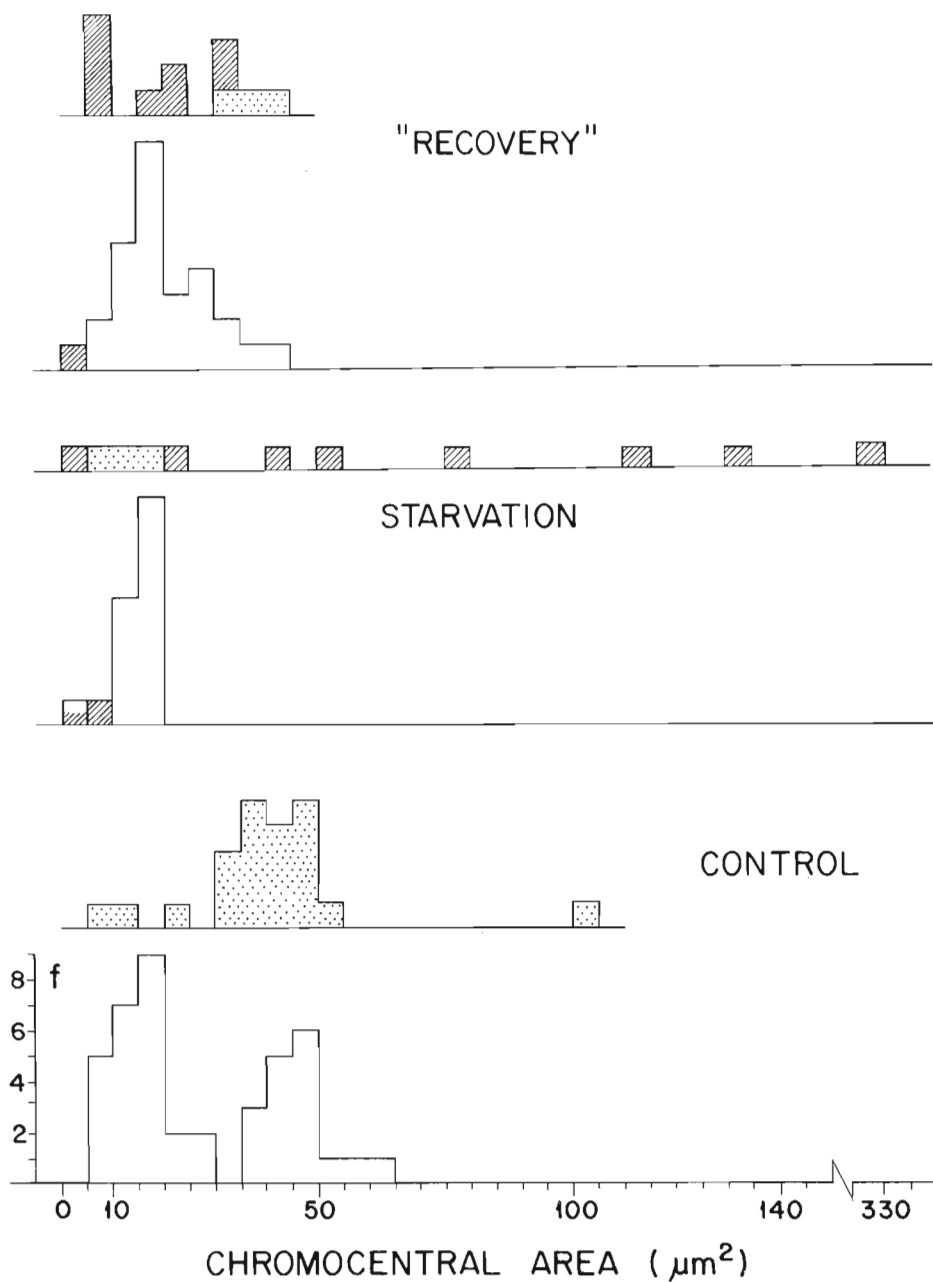


Figure 10 - Chromocentral areas in  $\mu\text{m}^2$ . f = frequency; open columns: single-chromocentered nuclei; dot-filled columns, multichromocentered nuclei; slant shaded columns, fused nuclei.

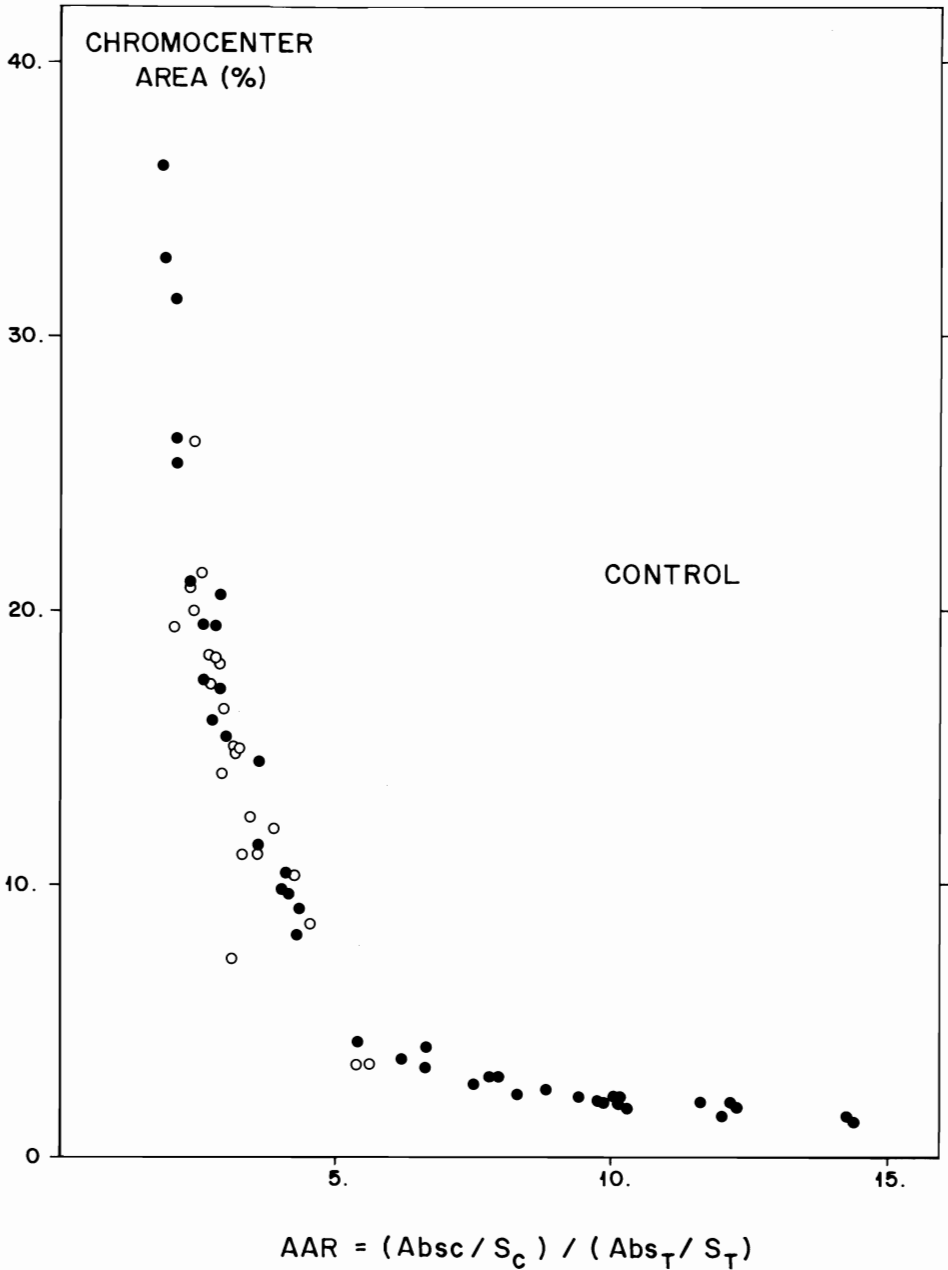


Figure 11 - Correlation of AAR and chromocentral areas (%) in fully-nourished specimens of *T. infestans*. ● = single-chromocentered nuclei; ○ = multichromocentered nuclei.

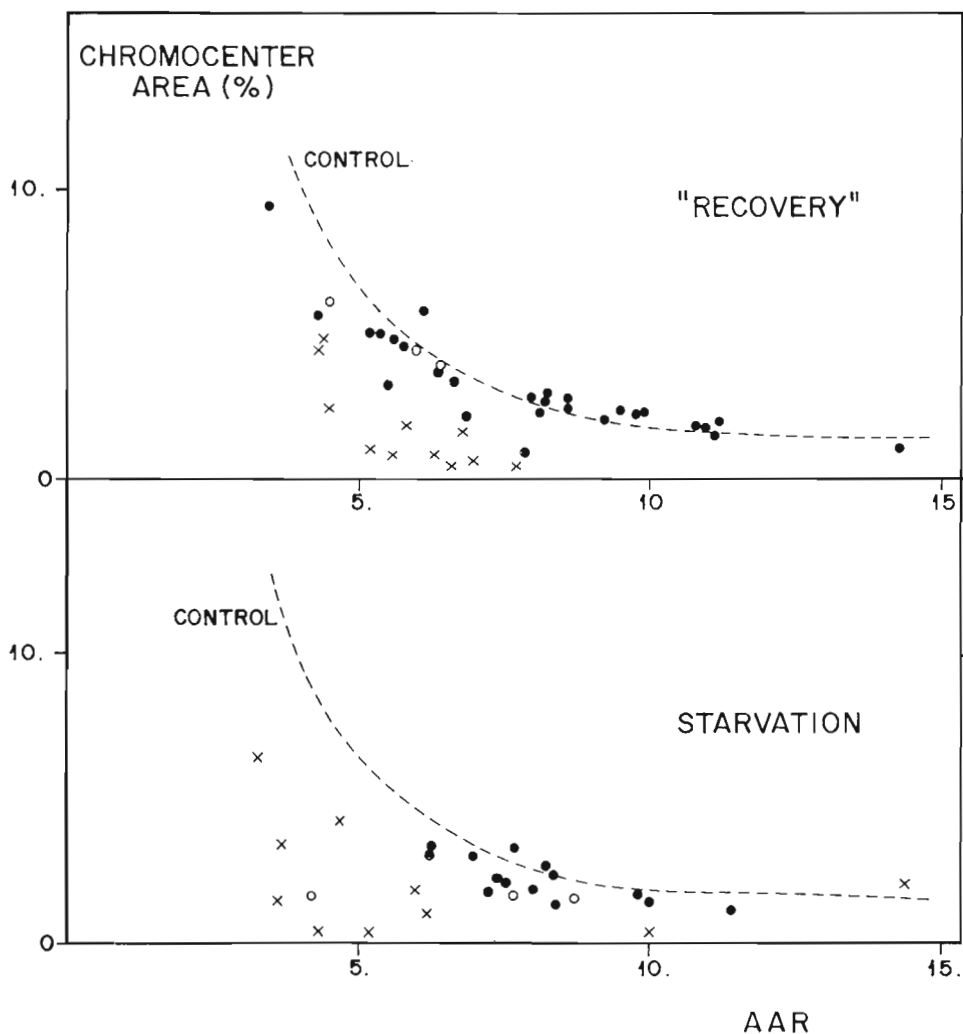


Figure 12 - Correlation of AAR and chromocentral areas (%) in starved and "recovered" specimens of *T. infestans*. x = fused nuclei; ● = single-chromocentered unfused nuclei; ○ = multichromocentered unfused nuclei.

should also occur in the insects starved for a long period, as ploidy degrees higher than 128 C could be found. 32C and 64C are the maximal ploidy degrees attained by nuclei of the Malpighian tubule cells of fully-nourished specimens of *T. infestans* through endoreplication (Mello, 1971, 1978). Furthermore, even considering that binucleate cells are a rule in Malpighian tubules of fully-nourished reduviids (Mello and Reccopimentel, 1987), fusion of two nuclei within a single cell could not give rise to a

nucleus with as high ploidy degree as that attained by some of the giant nuclei. Nuclear and cell fusions induced by starvation are likely to be favored by the presence of viruses which are often found in the blood-sucking hemipterans reared in the laboratory (Dolder and Mello, 1978).

It is worth mentioning that among several stressors to which *T. infestans* has been subjected in this laboratory (copper and mercury ions, starvation, antibiotics, fungal toxins) starvation appears to be the only one which induces nuclear fusion *in vivo*, possibly because this phenomenon requires a much longer stressing action to be elicited.

Since a fixed absorbance value was used as a "cut off" point for the discrimination of chromocentral Feulgen-DNA values and areas, the values of several parameters of the image analysis changed in part of the fused and unfused nuclei of the starved and "recovered" insects. Considering that absorbances below the "cut off" point were cleaned from the chromocenter image, their occurrence indicates changes in the packing state of the chromocentral heterochromatin. These changes are a consequence of a certain unravelling of the chromocentral heterochromatin, especially in those cases for which enlargement or no change in chromocentral area appeared concomitant with a decrease in Feulgen-DNA values.

Change in the heterochromatin packing state in response to starvation may represent an attempt to activate usually silent genes. Similar results have been reported for the same material in response to another stressor, copper ions (Kubrusly, 1984). Swollen heterochromatin zones have also been reported in other cell systems in response to heat shocks, viral and microsporidian infections, antibiotic action, and in certain cancer cells (Diaz *et al.*, 1969; Sandritter *et al.*, 1974; Simões *et al.*, 1975; Simões and Cestari, 1982).

Whether the unpacking state of the heterochromatin is related to the expression of *hsp* genes (Atkinson and Walden, 1985) is a matter for future study. Electron microscopy studies carried out by Mello and her co-workers (1989) have revealed that with extreme starvation the typical nucleolar zones rich in rRNA granules which usually surround the chromocenters of *T. infestans* are often replaced by a mass of structures resembling the perichromatin granules reported in rat liver cells subjected to hypothermal shock (Puvion *et al.*, 1977). According to Monneron and Bernhard (1969), the perichromatin granules would be carriers of mRNA in storage or transport form. Considering that the presence of these granules did not persist in nuclei of *T. infestans* starved specimens after feeding (Mello *et al.*, 1989) their appearance is probably related to a physiological change which affected the chromocentral heterochromatin due to starvation stress.

Among the several parameters used for the nuclear image analysis, correlation of AAR vs. condensed chromatin areas in percentages (Vidal, 1984) proved to be especially useful, as it discriminated graphically fused and even part of the unfused

nuclei of the starved and "recovered" specimens from nuclei of the control. In fact, it indicated that the relatively unpacked state of the chromocentral heterochromatin did not revert on cessation of the stressor direct action, maybe because of the severity of the stress.

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### RESUMO

Células epiteliais de túbulos de Malpighi provenientes de ninfas de 5<sup>o</sup> estadio de *Triatoma infestans* bem alimentadas, submetidas a um jejum prolongado e realimentadas após jejum ("recuperadas") tiveram seus núcleos estudados com microespectrofotometria de varredura e análise de imagem após reação de Feulgen. O objetivo foi correlacionar alterações nos fenótipos nucleares com mudanças em conteúdo Feulgen-DNA e/ou estrutura do cromocentro, em resposta ao "stress" do jejum. Foram encontrados núcleos semelhantes aos do controle, bem como núcleos fundidos e degenerados, nos espécimes em jejum e em "recuperação". Nos insetos em jejum comprovou-se que a fusão nuclear provocou o aparecimento de níveis de conteúdo de DNA de até 1024C, porém núcleos fundidos gigantes não foram observados nos insetos em "recuperação", possivelmente porque degeneraram. Com a análise de imagem demonstraram-se mudanças nos fenótipos nucleares envolvendo descompactação da área heterocromática em parte da população de núcleos fundidos e não fundidos dos insetos em jejum ou em "recuperação". O estado relativamente frouxo da heterocromatina não reverte, cessando a ação do agente estressante. Embora o jejum pareça ser um agente estressante especial para induzir fusão nuclear em células *in vivo* de *T. infestans*, o fenômeno de descompactação da heterocromatina, possivelmente uma tentativa de ativação de gene(s) silente(s) não pode ser considerado resposta específica ao jejum.

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