

JUVENILE HORMONE ACTION ON POLYPLOIDIZATION OF *Apis mellifera* FAT BODY

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ABSTRACT

The DNA content of fat body nuclei of *Apis mellifera* was assayed by scanning microspectrophotometry, after Feulgen staining. Several classes of nuclear ploidy were found in queen fat bodies. Polyploidy in drones was lower than in queens but higher than in workers.

The competence of *Apis mellifera* to synthesize DNA in response to juvenile hormone was analysed. An increase in ploidy was observed in workers (newly emerged adults) after topical application of Juvenile hormone III to 5th-instar larvae (L5F).

INTRODUCTION

Polyploidy, a form of genome amplification commonly occurs in organs that are hormone targets, such as the fat bodies of insects, which synthesize large amounts of protein. In some insects, polyploidization of the fat body is induced by juvenile hormone (JH). The effect of this hormone in inducing DNA synthesis and consequent polyploidization of fat body cells has been observed in *Locusta migratoria* (Nair *et al.*, 1981; Irvine and Brasch, 1981), *Schistocerca gregaria* (Kooman and Nair, 1987), *Coccinella septempunctata* (Quan and Chen, 1983), and *Aedes aegypti* (Ditmann and Hagedorn, 1984).

Several studies on tissue polyploidization in drone, worker and queen honey bees have been made. A brief account of some aspects of these studies was reported by Woyke and Krol-Paluch (1985). However, in these studies there are no references about

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the extent of ploidy in newly emerged queens, workers and drones fat bodies. Nor are there references about the possible effect of JH III on fat body polyploidization of these bees.

In this study, we have examined the fat body ploidy classes of the different castes and sexes, and the influence of JH III on polyploidization, using microspectrophotometry of Feulgen stained preparations.

MATERIALS AND METHODS

JH treatment of workers

Three colonies of africanized *Apis mellifera* were used. Fecundated *Apis mellifera* queens were confined on combs for six hours, so that they would oviposit. This permitted obtaining a reasonable number of workers (approximately 80 to 200) that would develop in a synchronized manner.

Workers in the L5F (feeding larvae) and prepupal phases (seven and ten days of development, respectively, considering oviposition as day 0) were treated with one μ l of JH III solution (one μ g of JH III dissolved in one μ l acetone) applied topically. Control groups received topical applications of one μ l of acetone.

Prepupae were removed from brood combs, placed on Petri dishes and treated with JH III. Development was allowed to progress in incubators at 34°C and 80-90% relative humidity for four days (pink-eyed pupa phase) or until emergence.

L5F larvae were treated in the brood combs and returned to the hives immediately after treatment. Larvae feed and therefore placement in an incubator would impair their development. Three days before worker emergence, i.e., during the black-eyed pupal phase, the bees were removed from the comb and transferred to an incubator where development progressed until emergence under the same temperature and relative humidity conditions as described above.

The fat bodies of workers, pink-eyed pupae and newly emerged bees treated or not with JH during the prepupal phase, and the fat bodies of newly emerged workers treated during the L5F phase were excised and prepared for microspectrophotometry (Table I).

JH treatment of drones

Drones were obtained from non-fecundated queens. As also for workers, oviposition was restricted to a defined period of time, which in this case was 24 hours.

Drones in the prepupal phase were removed from brood combs and treated with one μ l of JH III solution, prepared as cited above. Development until emergence was allowed to progress in an incubator under the conditions mentioned above.

Table I - Developmental phases during which workers and drones received JH treatment and phases during which the fat bodies were excised and prepared for microspectrophotometry.

| <i>Apis mellifera</i> | JH treatment | Fat body extraction |
|-----------------------|--------------|---------------------|
| Workers | Larva (L5F) | Newly emerged adult |
| | Prepupa | Pink-eyed pupa |
| | Prepupa | Newly emerged adult |
| Drones | Prepupa | Newly emerged adult |

After emergence, the fat body was excised from the dorsal cuticle and prepared for microspectrophotometry (Table I).

Queens

Queens were produced by transfer of L1 worker larvae (three days of development after oviposition) to queen cups containing royal jelly. On the day before emergence, the queen cells were transferred from the queen finishing colonies to an incubator. The fat bodies of the newly emerged queens were excised and prepared for microspectrophotometry.

Preparation of fat body for cytospectrophotometry

The fat bodies of queens, workers and drones were excised in insect Ringer solution, transferred to gelatinized glass slides and squashed with the help of a coverslip. Slides containing fat body were immersed in liquid nitrogen to unglue and remove the coverslips.

The tissue was fixed in 3:1 ethanol-acetic acid for 25 minutes and stored in 70% ethanol at 4°C before being submitted to the Feulgen reaction.

Hydrolysis was performed at 5N HCl, at 30°C for 20 minutes. This time was selected after a microphotometric test carried out to compare the effects of different hydrolysis times on the intensity of DNA staining. After hydrolysis the material was washed with ethanol (Larson and Sauaia, 1980) for three minutes, stained with Schiff reagent for 40 minutes, washed three times with sulfurous water, dehydrated, and mounted on Canadian balsam. The absorbance of the nuclei of fat body cells was determined at 570 nm using a Zeiss scanning spectrophotometer and is reported in arbitrary units. A 1 µm step, 0.5 µm aperture and a 40x objective were used to scan each nucleus.

DNA content was determined in five to ten trophocytes of the fat body from each pink eyed pupa and from each newly emerged worker, drone and queen, for a total of 1469 trophocytes and 281 enocytes.

We used the Kolmogorov-Smirnov test ($\alpha = 0.01$) to compare the frequency distributions obtained from cytospectrophotometric readings of trophocytes of bees treated or not with JH III. A test for normality was applied when deviation from unimodality was suspected.

RESULTS

Determination of the DNA content of enocytes

DNA measurements in fat body enocyte nuclei provided the reference value for interpretation of the microspectrophotometric readings obtained for trophocytes. Enocytes from newly emerged workers showed a unimodal distribution, with mean absorbance value equal to 27.4 ± 4.5 arbitrary units for $N = 168$ (Figure 1). These mean absorbance indicated the 2C value.

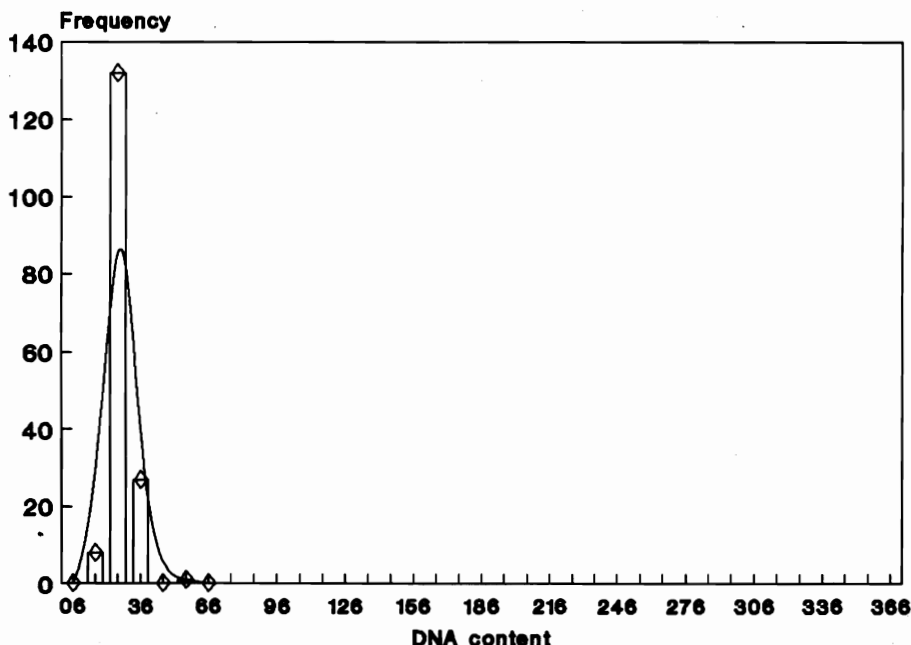


Figure 1 - Frequency distribution of fat body enocyte nuclei from newly emerged *Apis mellifera* workers. Ordinate: absolute frequency of the determinations. Abscissa: absorbance at 570 nm, in arbitrary units of Feulgen stained preparations.

Determination of the DNA content of trophocytes

Worker pupae: JH treatment during the prepupal phase

Figures 2A and B show the frequency distributions of trophocytes from pink eyed worker pupae treated with JH III or with acetone during the prepupal phase. These distributions characterize two trophocyte classes according to DNA content, suggesting genome duplication. The distribution of nuclei ($N = 227$) in the JH-treated group was not significantly different from that ($N = 95$) in the untreated group (Kolmogorov-Smirnov test, $DN = 0.093$, $P > 0.5$).

The peaks of highest frequency in Figures 2A and 2B did not exactly coincide with the 2C value established using the encytes. This result may reflect partial genome replication of most trophocytes. This small difference may also be attributed to the fact that the intensity of Feulgen staining may be influenced by the extent of chromatin condensation (Lorick, 1970), which differed between encytes and trophocytes. Whatever the case, the peaks clearly showed two degrees of ploidy both in JH-treated and untreated pupae.

Newly emerged workers: treatment with JH during the prepupal phase

Figures 3A and B show the frequency distributions of the trophocytes from newly emerged workers treated or not with JH III during the prepupal phase. As also observed in pupae (Figures 2A, B), the distributions indicate two trophocyte classes according to DNA level. Trophocytes ($N = 116$) from treated group did not show significantly different frequency distributions from untreated group ($N = 163$) according to Kolmogorov-Smirnov test, $DN = 0.050$, $P > 0.5$.

A higher frequency of trophocytes with values close to 4C was observed in newly emerged workers (Figure 3A and B) than in pupae (Figure 2A and B). This result indicates that DNA replication occurs in most trophocytes between the pink-eyed pupal phase and emergency, with a doubling of the degree of ploidy.

Newly emerged workers: JH treatment during the L5F phase

Figure 4A shows the frequencies of trophocytes ($N = 261$) of newly emerged workers treated with JH III during the L5F phase, and Figure 4B shows the frequency distribution of trophocytes ($N = 169$) of the control group.

As also observed in pupae (Figures 2A and B), and newly emerged workers treated (with JH III or acetone) during the prepupal phase (Figures 3A and B), the trophocytes of workers treated during the L5F phase (Figure 4A and B) were distributed

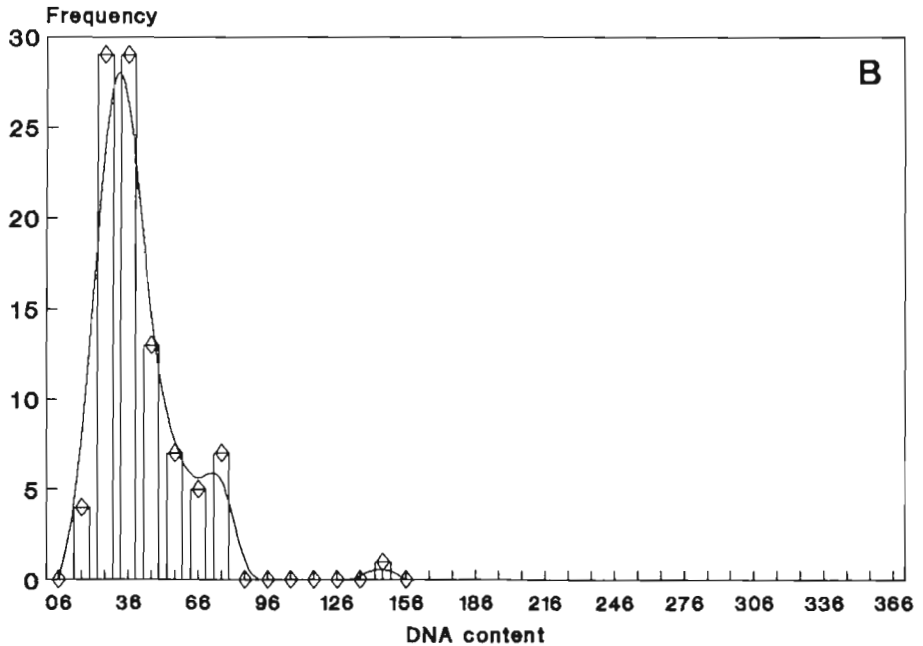
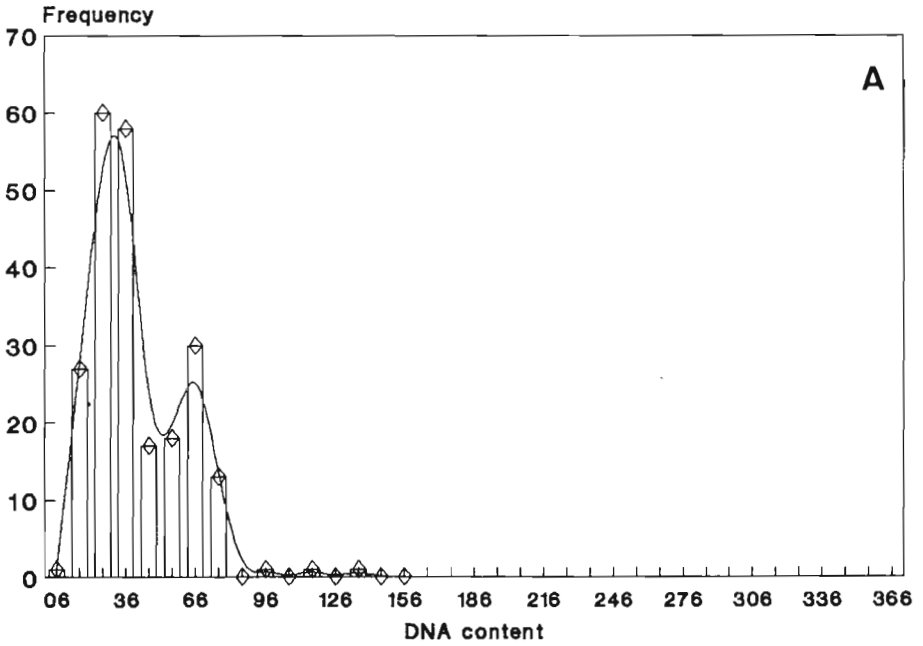


Figure 2 - Frequency distributions of DNA classes in fat body trophocytes from pink-eyed *Apis mellifera* worker pupae, treated (A) and not treated (B) with JH III during prepupae phase. Ordinate: absolute frequency of the determinations. Abscissa: absorbance at 570 nm, in arbitrary units, of Feulgen stained preparations.

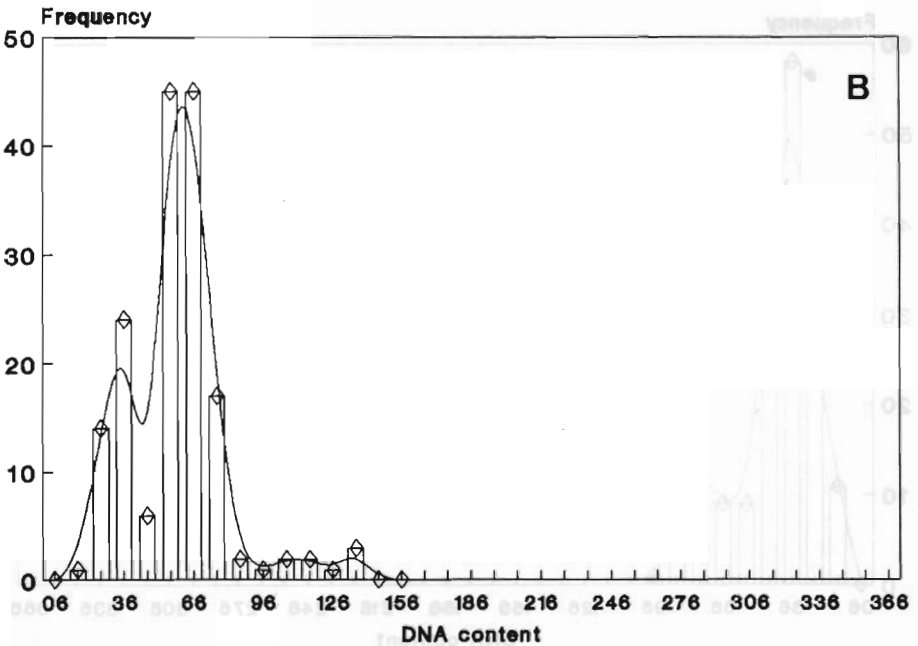
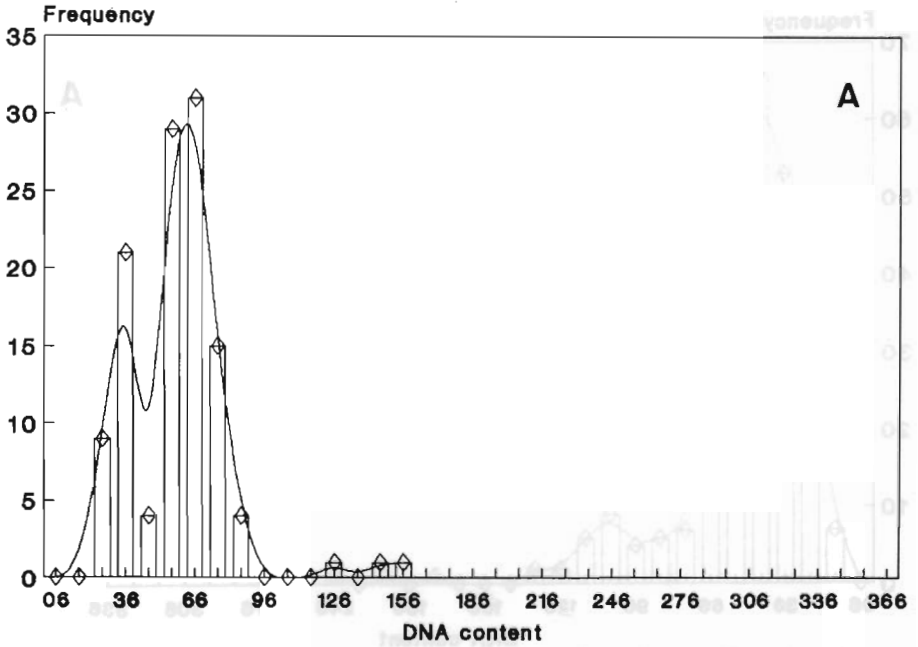


Figure 3 - Frequency distributions of DNA classes in fat body trophocytes from newly emerged *Apis mellifera* workers, treated (A) and not treated (B) with JH III during prepupae phase. Ordinate: absolute frequency of the determinations. Abscissa: absorbance at 570 nm, in arbitrary units, of Feulgen stained preparations.

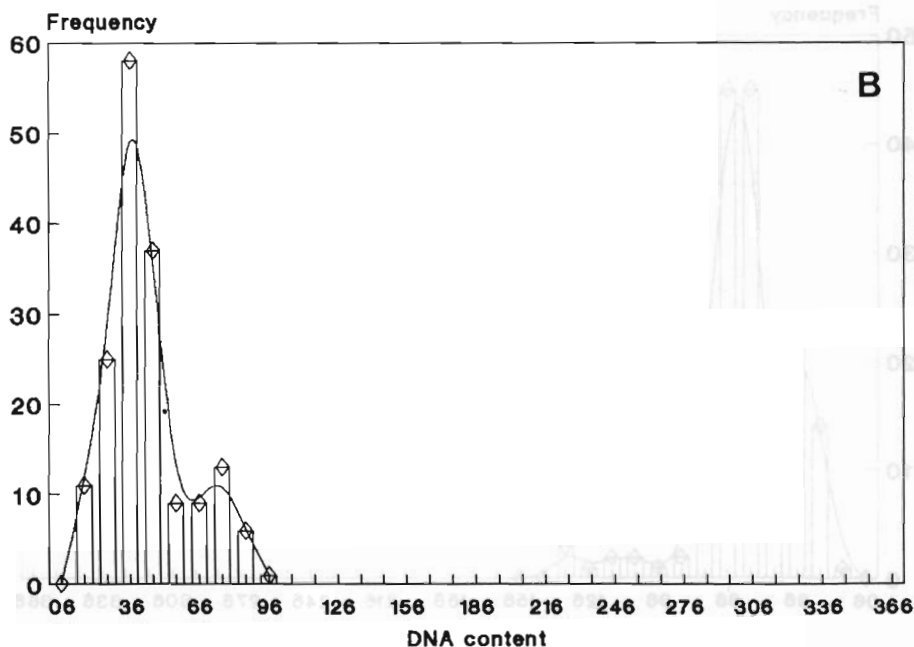
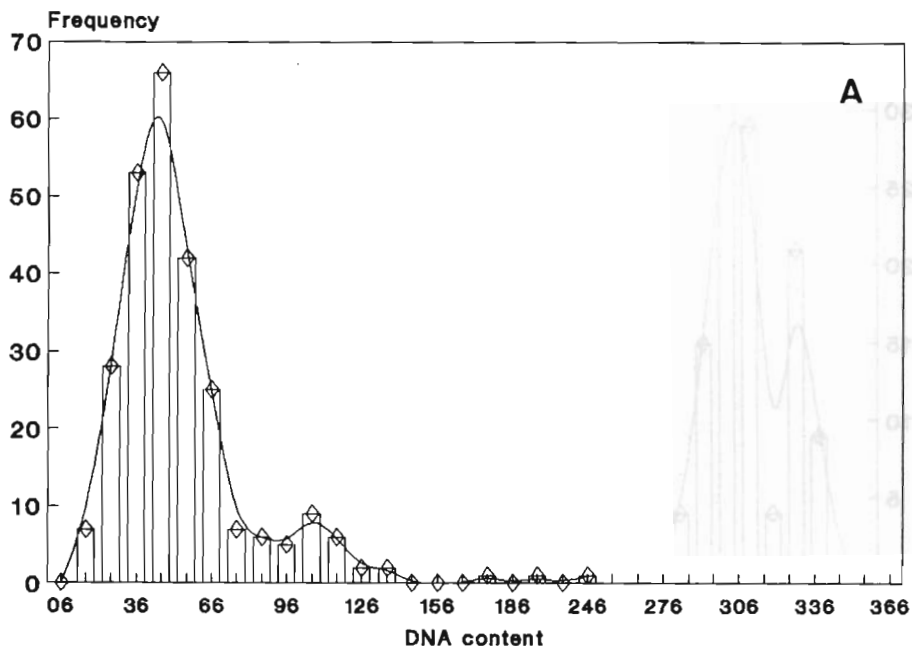


Figure 4 - Frequency distributions of DNA classes in fat body trophocytes from newly emerged *Apis mellifera* workers, treated (A) and not treated (B) with JH III at the L5F phase. Ordinate: absolute frequency of the determinations. Abscissa: absorbance at 570 nm, in arbitrary units, of Feulgen stained preparations.

into two classes of ploidy. However, in JH-treated workers (Figure 4A), the frequency distribution was shifted to the right when compared with the distribution observed in untreated workers (Figure 4B). This significant difference (Kolmogorov-Smirnov test, $DN = 0.260$) demonstrates the effect of JH III on trophocyte DNA synthesis, but only when the hormone is applied during the L5F phase.

When comparing curves 3A and 3B to curves 4A and 4B higher trophocyte frequencies are observed around the 2C value both when JH III treatment is applied during the L5F phase and in the controls (Figure 4A and B). The inverse occurs when treatment is performed during the prepupal phase (Figure 3A and B). This difference may be explained by the possible effect of bee handling and of introducing substances into the comb during a phase (L5F) characterized by physiological changes leading to metamorphosis. Honey bees are highly susceptible to manipulation, which may cause physiological, biochemical and behavioral changes. These changes usually cause a "delay" in ontogenetic development (Bitondi and Simões, unpublished data), which results in decreased frequency of trophocytes that undergo genome replication (Figure 4A and B).

Shigematsu *et al.* (1978) observed an increase in DNA in the silk gland of *Bombyx mori* after treatment with a synthetic JH analogue. However, this increase occurred only under suboptimal growth conditions and was associated with prolongation of larval instar, which may have been the cause of the "delay" in development mentioned above.

Queens

Figure 6 shows the frequency distribution of trophocytes ($N = 202$) in newly emerged queens. The formation of a new class of ploidy can be seen, with values between 8C and 16C, thus demonstrating a more intense polyploidization process in queens than in workers.

Newly emerged drones: JH treatment during the prepupal phase

The frequency distributions of trophocytes in drones according to DNA content are illustrated in Figure 5A and B. The distributions did not differ significantly between the trophocytes ($N = 110$) of JH III-treated drones and the trophocytes ($N = 126$) of untreated drones (Kolmogorov-Smirnov test, $DN = 0.075$, $P > 0.5$).

In drones, the highest trophocyte frequency was observed around the 4C value, although there were also trophocytes with values similar to 8C, a ploidy class not observed in workers. Therefore, the fat bodies of drones have higher ploidy values than those detected in workers (Figure 3A and B) and lower than those observed in queens (Figure 6).

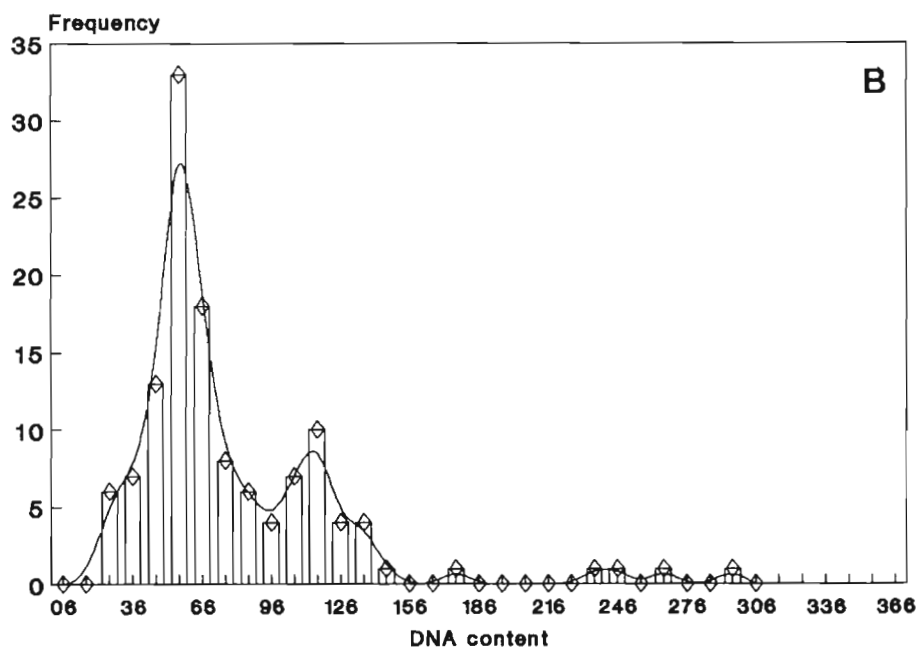
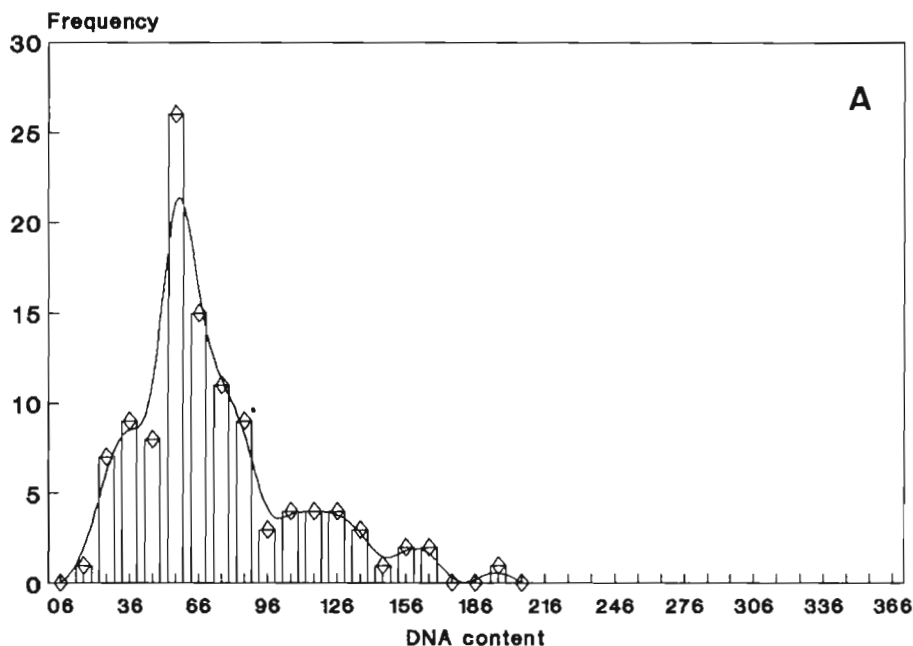


Figure 5 - Frequency distributions of DNA classes in fat body trophocytes from newly emerged *Apis mellifera* drones treated (A) and not treated (B) with JH III during the prepupal phase. Ordinate: absolute frequency of the determinations. Abscissa: absorbance at 570 nm, in arbitrary units, of Feulgen stained preparations.

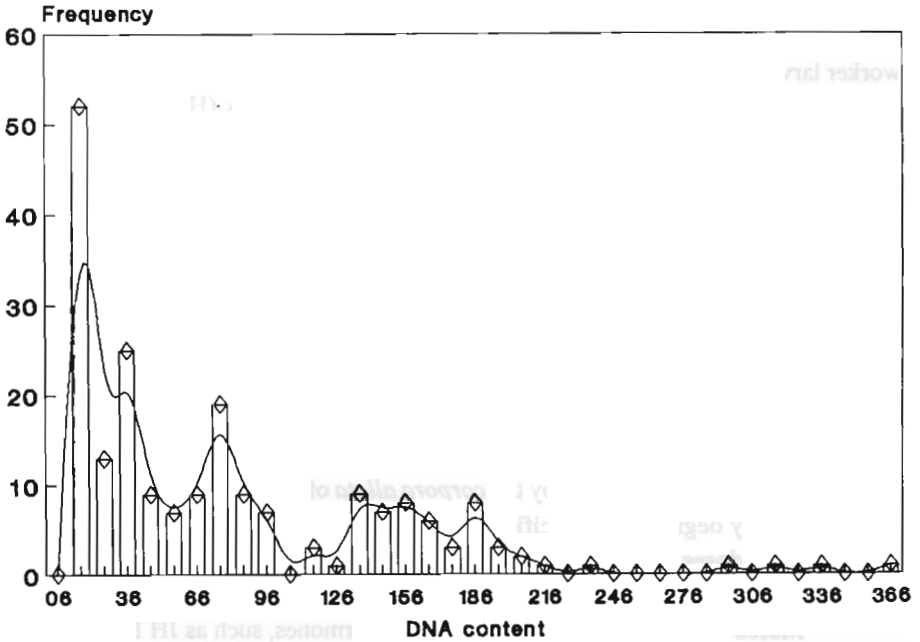


Figure 6 - Frequency distribution of DNA classes in fat body trophocytes from newly emerged *Apis mellifera* queens. Ordinate: absolute frequency of the determinations. Abscissa: absorbance at 570 nm, in arbitrary units, of Feulgen stained preparations.

DISCUSSION

DNA measurements in trophocytes of newly emerged workers treated with JH III during the L5F phase showed the inducing action of the hormone on the polyploidization of the fat body. Although exogenous JH III did not cause the appearance of new ploidy classes, it significantly shifted the frequency distribution, showing that a larger number of trophocytes underwent DNA replication in treated bees (Figure 4A and B).

When treatment was performed during the prepupal phase there was no change in the DNA content of trophocytes: the frequency distribution of these cells, according to DNA content, observed in the treated workers did not differ from that observed in the controls (Figure 3A and B).

The intensification of the polyploidy process when treatment is performed during the L5F phase and not during the prepupal phase may be explained by the differential sensitivity to JH of the specific phases of ontogenetic development of *A.*

mellifera. Wirtz (1973) detected this period of sensitivity to the action of JH when he obtained greater success in transforming workers into queens by treating 3 to 3.5-day worker larvae with JH. The period of sensitivity of *A. mellifera* has been assigned to the L5F phase and occurs simultaneously with the detected JH peak (Hartfelder, 1990).

With one μg of JH III per larvae we could detect a measurable response, i.e., an inducing effect on fat body polyploidization. This is a small dose when compared to the 3-14 μg used by Goewie and Beetsma (1976) in order to induce queen traits in treated workers. According to these authors, hardly any worker adults with queen characteristics were obtained after application of one μg of JH III to the larvae. We also did not detect queen traits in workers treated with this dose. The small dose utilized also would explain why the frequency distributions differ widely between queens and workers treated during the L5F phase. The increasing ploidy detected in these workers is very discrete when compared to the ploidy observed in queen fat bodies. This discrete effect maybe can be related to the small JH III dose utilized. Here we have to point on the fact that the JH III is a natural hormone, synthesized by the *corpora allata* of *A. mellifera*. As a consequence, it can be rapidly degraded by specific esterases. Then, perhaps the application of higher and repeated doses of this hormone, may permit to obtain trophocyte frequency distributions less different between queens and treated workers.

Moreover, the utilization of non-natural hormones, such as JH I and II, or even the use of synthetic hormones may permit a more prolonged action which turns the effect more evident. Barbosa and Simões (unpublished results) have observed morphological alterations and changes in the protein patterns obtained by isoelectric focusing in the fat body of worker larvae cultured *in vitro* in the presence of JH I. The effect of JH III on this tissue using the same methods was much less evident. In a similar manner, JH I is more effective in inducing queen traits when applied topically to worker larvae than JH III (Goewie and Beetsma, 1976, cited by Severson *et al.*, 1989).

We observed a higher degree of polyploidization in drones than in workers. Risler (1954) showed that drones are haploid only up to the first larval instar. In the subsequent instars, the polyploidization process of drone tissues is intense. Consequently, the degree of ploidy observed in the last larval instar of the drone is identical to that observed in workers. Similarly, Woyke and Krol-Paluch (1985) observed that the DNA content of different tissues (ventricle, gut, silk glands, and fat body) of worker larvae (four days) is quite similar to that of haploid drones, indicating that the rate of polyploidization is greater in drones than in workers if we consider that, at the beginning of development, drones are haploid and workers diploid. Thus, the DNA content increases more rapidly in drones than in workers during the larval stage. Woyke and Krol-Paluch (1985) also studied the pattern of ploidy of newly emerged workers and drones and noted that the degree of ploidy varied according to the tissue under study. The ventricle, gut and Malpighian tubules of workers contain larger amounts of DNA than the

corresponding tissues of drones. However, the post-cerebral glands of drones have DNA amounts that are significantly greater than those in the glands of workers and similar to those of queens. Our data show that the fat bodies of drones have an intermediate level of ploidy, which is greater than that of workers but lower than that of queens.

Polyploidy occurs in many types of cells which specialize in the production of large amounts of protein, such as the cells of insect fat bodies. In *L. migratoria*, the polyploidy of the fat body is related to an increase in vitellogenin (Vg) synthesis in this tissue (Irvine and Brasch, 1981; Nair *et al.*, 1981). One-day old males and females of *L. migratoria* have very similar degrees of ploidy, although on the 15th day of life males present less replication than females (Nair *et al.*, 1981). The magnitude of the increase in ploidy is greater in females both in absolute and relative terms (Irvine and Brasch, 1981). However, the fat body of *Locusta* males, despite polyploidy, does not synthesize Vg.

The frequency distribution of *A. mellifera* queen trophocytes indicates the occurrence of many more cycles of DNA replication than observed in workers during the same phase. This high degree of ploidy in queens suggests a relationship with the intensification of protein synthesis, and perhaps also of Vg, the major protein in hemolymph.

Although the ontogenetic pattern of Vg production is similar in drones and workers, the absolute amount of this protein in hemolymph is much smaller in drones than in workers (Trenczek *et al.*, 1989; Engels *et al.*, 1990). Therefore the greater process of fat body polyploidization in drones than in workers does not seem to be linked to Vg synthesis.

ACKNOWLEDGMENTS

We are grateful to Dr. H. Sauaia for help with the spectrophotometric measurements, to Dr. J.A. Lobo for the Kolmogorov-Smirnov analysis and to Mr. J.J. dos Santos for help with breakeeping work.

Publication supported by FAPESP.

RESUMO

O teor de DNA das células do corpo gorduroso de *Apis mellifera* foi medido por micro-espectrofotometria após hidrólise com HCl 5N e coloração com reagente de Schiff. Analisamos os padrões de ploidia das rainhas, zangões e operárias e a competência das operárias para sintetizar DNA em resposta ao hormônio juvenil. Um aumento significativo no número de núcleos com maior teor de DNA foi observado em operárias recém emergidas após aplicação tópica de hormônio juvenil III às larvas em fase L5F. Tal aumento não se verificou quando esse hormônio foi aplicado à fase de prepupa.

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(Received September 17, 1991)