Micro-interferometry of Insect Polyploid Nuclei

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Introduction

Interference microscopy has enabled investigators to determine the weight of unstained and even living cells and cellular components by precise optical means and has also provided a direct image of structural details with excellent and controllable contrast and high resolution (Mellors 1957). By interference microscopy measurements of dry mass of genetic material in sperm nuclei of the mouse, the rat and the guinea pig and of the different stages of grasshopper spermatogenesis have been reported (Mellors and Hlinka 1955, Watkins 1961).

Detection of polyploid nuclei in quantitative cytological analysis has used the number of chromosomes and, when this cannot be established, estimates of the nuclear DNA from cytophotometry and the nucleus size from caryometry. Micro-interferometry procedures for determination of dry mass in polyploid somatic nuclei had been only used in the study of neoplastic cells in mouse tumours (Mellors *et al.* 1955, Mellors 1957) and of isolated diploid and tetraploid nuclei of adult rat liver (Bibbiani *et al.* 1969) and were considered of high precision, when comparisons were made with cytophotometric data for DNA.

Development of polyploidy in silk glands and Malpighian tubes of insects has been demonstrated in terms of DNA content per nucleus and nuclear size. In Hymenoptera the larval Malpighian tubes of *Melipona quadrifasciata* have polyploid nuclei during the post-embryonic development; DNA replications stop in the 3rd instar, while the nuclear volumes enlarge until the 4th instar (Mello and Takahashi 1969). DNA contents and nuclear volumes in the silk glands of the same bee increase until the 4th larval instar, but the increase in nuclear volume is greater than that in DNA content (Mello *et al.* 1970). The Malpighian tubes of *Melipona quinquefasciata*, a stingless bee which has germinative polyploid cells, show the same DNA content per nucleus as those of *Melipona quadrifasciata* (Mello and Silveira 1970).

In the present work some phenomena of somatic polyploidy in bees are investigated by micro-interferometric procedures. The dry masses of the nuclei are determined, and comparisons are made with reported karyometric and cytophotometric data, mainly in cases in which increases in nuclear volumes are not in accordance with DNA increases.

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Material and methods

Worker stingless bees of *Melipona quadrifasciata anthidioides* Lep. and *Melipona quinquefasciata* Lep. were used.

The Malpighian tubes of *Melipona quadrifasciata* were studied in the five larval instars and prepupa, and the silk glands in the 3rd and 5th larval instars. The nuclei of the distal and proximal portions of the silk glands in the 3rd instar were investigated, since evidence of differential nuclear activity of parts of this organ has been reported (Mello *et al.* 1970). The Malpighian tubes and silk glands of *Melipona quinquefasciata* were studied in the last larval instar and compared with those of *Melipona quadrifasciata*.

The organs were dissected in 0.75% saline solution and left for 3–5 minutes in distilled water, which ruptures cells and liberates nuclei and this was observed with the interference microscope. The nuclei were then immersed in Carlson's medium (Shaw 1956) in order to have their dry masses determined after 20 minutes.

Observations were made with a Zeiss photomicroscope equipped for polarization and interference (Pol-Interferencial) with monochromatic light ($\lambda=546$ nm) for an U.V. source.

Dry mass in grams of material was given by the formula

$$m = \Phi_m \frac{A}{\chi} + (\mu_m - \mu_w)t \frac{A}{\chi}, \text{ where}$$

Φ_m is the optical path difference measured relative to the medium of refractive index μ_m ; A , the area of the nucleus (cm^2); $\mu_m = 1.336$ (refractive index of Carlson's medium); $\mu_w = 1.333$ (refractive index of distilled water); t , the nucleus thickness, and $\chi (=100\alpha) = 0.18$ (Davies *et al.* 1954).

Measurement of the optical path difference was computed with the Senarmont's method, in which compensation is determined by a rotatory analyser. The nuclear diameter was measured with a delicate rotatory vernier scale inserted in a special eye-piece (Leitz).

Errors in the dry mass determinations due to the assumption of an average α value of 0.0018 do not exceed $\pm 10\%$ in cells containing mainly solutions or gels of proteins, lipoproteins, and nucleic acids (Davies *et al.* 1954). This error could arise only if the structure consisted entirely of only one of these substances. It would be less in a mixture of these substances as in the nuclei. Since the main component of nuclei is often protein, 0.0018 is an accurate value for α (Hale 1958).

Nucleic acids and histones were extracted from nuclei of silk glands and Malpighian tubes in the last larval instar of *Melipona quadrifasciata* for calculation of their part in the dry mass of the nuclei. Nucleic acids were extracted in a 5% aqueous solution of TCA for 2 hours at 90°C (Davies *et al.* 1957). The completeness of extraction with TCA was checked by Einarson's chromalum-gallocyanin staining (1951). Digestion of histones was investigated with a 0.5% trypsin solution in 0.75% saline solution for 12 hours at 36°C (modified from Davies *et al.* 1954). A collagen bundle was used as a control for trypsin action.

All dry mass calculations were performed on an IBM-360 computer.

Original data were placed on a scale of values in geometric progression since they have a long-normal distribution and this kind of scale avoids the search for logarithms (Ferreira *et al.* 1967).

Results

The nuclei of the Malpighian tubes of *Melipona quadrifasciata* in the larval instars and prepupa had the dry masses plotted as frequency histograms (Fig. 1) and these data may be compared with Mello and Takahashi's (1969) karyometric and cytophotometric values on somatic polyploidy (Table 1). The last doubling of nuclear volume and dry mass occurs in the 4th larval instar but is not accompanied by an increase in DNA content.

Table 1. Summary of data of the nuclei from the larval Malpighian tubes of *Melipona quadrifasciata*

Stages	1st instar	2nd instar	3rd instar	4th instar	5th instar	Prepupa
Nuclear dry masses ($D \pm Sd$) $10^{-11}g$	4.18 ± 2.76	12.62 ± 6.16	35.34 ± 12.10	70.75 ± 19.69	77.90 ± 10.17	78.60 ± 21.28
Nuclear dry masses after hot TCA ($D \pm Sd$) $10^{-11}g$	—	—	—	—	44.76 ± 16.02	—
Per cent loss after hot TCA	—	—	—	—	42.59	—
Nuclear dry masses after trypsin ($D \pm Sd$) $10^{-11}g$	—	—	—	—	67.25 ± 10.26	—
Per cent loss after trypsin	—	—	—	—	13.74	—
Doublings in nuclear dry masses	y, 2y, 4y, 8y	4y, 8y, 16y	16y, 32y	64y	64y	64y
Doublings in nuclear volumes according to Mello and Takahashi (1969)	4x, 8x, 16x, 32x	16x, 32x	16x, 32x	16x, 32x, 64x	16x, 32x, 64x	16x, 32x, 64x
DNA replica- tions according to Mello and Takahashi (1969)	4n, 8n, 16n	16n, 32n	16n, 32n	16n, 32n	16n, 32n	16n, 32n

Higher values of dry masses are seen in the distal portion of the silk glands of *Melipona quadrifasciata* in the 3rd larval instar (Fig. 2) and this is in accordance with the two main ploidy degrees in terms of DNA content (8n and 16n) and nuclear volumes assumed by Mello *et al.* (1970). In the 5th larval instar most of the values are placed in an interval range higher than that found in the distal 3rd instar (Fig. 3)

Fig. 1. Frequency histograms of dry masses of nuclei from the larval Malpighian tubes (*Melipona quadrifasciata*). Arrows indicate the average of the distributions. L₁ to L₅ = larval instars PP = prepupa — = dry mass of nuclei after trypsin treatment - - - - = dry mass of nuclei after hot TCA treatment

Fig. 2. Frequency histograms of dry masses of proximal and distal nuclei from the silk glands in the 3rd larval instar (*Melipona quadrifasciata*).

Fig. 3. Frequency histograms of dry masses of distal nuclei from the silk glands in the 3rd and 5th larval instars (A) and of the 5th instar nuclei after trypsin (B) and hot TCA (C) treatments (*Melipona quadrifasciata*).

Fig. 4. Frequency histograms of dry masses of the nuclei from Malpighian tubes (MT) and silk glands (SG) in the 5th larval instar (*Melipona quadrifasciata* and *M. quinquefasciata*).

Table 2. Summary of data of the nuclei from the silk glands of *Melipona quadrifasciata*

Larval instars	3rd		5th
Portions of the silk glands	Proximal	Distal	Distal
Nuclear dry masses (D±Sd) 10 ⁻¹¹ g	52.80±16.04	71.71±19.19	137.90±32.51
Dry mass after hot TCA (D±Sd) (10 ⁻¹¹ g)	—	—	72.47±18.18
Per cent loss after hot TCA	—	—	47.45
Dry mass after trypsin (D±Sd) (10 ⁻¹¹ g)	—	—	89.76±14.09
Per cent loss after trypsin	—	—	34.92
Doublings in nuclear dry masses	4y, <u>8y</u> ¹	8y, 16y	16y, <u>32y</u>
Doublings in nuclear volumes according to Mello <i>et al.</i> (1970)	4x, 8x	8x, <u>16x</u>	16x, 32x, <u>64x</u>
DNA replications according to Mello <i>et al.</i> (1970)	4n, <u>8n</u>	8n, 16n	<u>32n</u> , 64n

¹ Underlined values correspond to the most populated interval.

and correspond to $32n$ interval according to cytophotometric data for DNA reported by Mello *et al.* (1970) (Table 2).

The dry mass of the nuclei from Malpighian tubes and silk glands of *Melipona quinquefasciata* are slightly larger than those of *Melipona quadrifasciata* (Fig. 4). The values of arithmetic mean with standard deviation for these organs are:

Malpighian tubes:	$80.23 \pm 25.48 \times 10^{-11} \text{g}$ (<i>M. quinquefasciata</i>)
	$77.90 \pm 20.17 \times 10^{-11} \text{g}$ (<i>M. quadrifasciata</i>)
Silk glands:	$144.40 \pm 42.45 \times 10^{-11} \text{g}$ (<i>M. quinquefasciata</i>)
	$137.90 \pm 29.51 \times 10^{-11} \text{g}$ (<i>M. quadrifasciata</i>)

The dry masses of the nuclei from Malpighian tubes and silk glands of *Melipona quadrifasciata* after hot TCA and trypsin treatments were determined in the 5th instar. Since the main mode of the distributions before and after treatments is placed within the same distribution range as the arithmetic mean values, the percentage of losses was calculated by using the arithmetic means of this instar (Tables 1 and 2).

Discussion

Increases in DNA content and nuclear volumes in the Malpighian tubes of *Melipona quadrifasciata* have been reported to be proportional each other until the 3rd larval instar, when DNA replications stop but the nuclear volumes duplicate once more (Mello and Takahashi 1969). This last enlargement of nucleus size is accompanied by an increase in the dry mass of the nucleus, and from the duplication values one can assume that the dry mass is in accordance with data from nuclear volume/DNA ratio (Table 1).

Such variations in nucleus enlargement have been attributed to non-histonic proteins (Mellors 1957, Alfert 1959). According to Schreiber *et al.* (1966) an extra doubling of nuclear volume would be related to a double amount of acidic proteins which would act as de-repressors of genetic loci, by displacing histones and allowing RNA transcription. Since histones are indeed inhibitors of gene function, the displacement of these inhibitors by non-histonic proteins (or RNA) would influence gene activity (Markert, 1965, *in* Schreiber *et al.* 1966). In *Chironomus* salivary glands acidic proteins have also been reported in the transport of RNA from chromosome to cytoplasm (Stevens and Swift 1966, Berendes 1968). There is a trypsin-resistant, DNase sensitive core inclosed in a trypsin-sensitive sheath in honey-bee chromatin fibers (DuPraw 1965). Presumably histones constitute a significant part of the sheath proteins, since they are known to be associated quantitatively with chromosomal DNA and to be synthesized when DNA is synthesized. The amount of histone, however, is lesser than that expected on the basis of total mass of the sheath. The bulk of the sheath protein is thought to be acidic, which is supported by the fact that isolated metaphase chromosomes are stabilized in acid solution (Chorazy *et al.* 1963, DuPraw 1965). Therefore, one can assume that most of the remaining nuclear dry mass of nuclei from *Melipona quadrifasciata* Malpighian tubes is due to acidic proteins.

Values of dry masses duplications in the nuclei of *Melipona quadrifasciata*

silk glands are in accordance with the endopolyploid gradient regarding distal and proximal portions of the organ (3rd instar). Since the main values of nuclear volume duplications in the 5th instar (64x) are larger than most of DNA contents (32n) and dry masses (32y), it is suggested that there is a high hydration level of the nuclear material of the silk glands in this stage. Values of dry masses of the silk glands in the 5th instar are twice those of the Malpighian tubes in the same instar (Fig. 3).

Material extracted with hot TCA or trypsin allow us to assume that there are 35.31×10^{-11} g of nucleic acids in the nuclei of the Malpighian tubes of *Melipona quadrifasciata* in the last larval instar (42.59% to total dry mass of the nuclei) and 10.75×10^{-11} g of histones (13.74%). In the silk glands of the last larval instar of the same bee there are 65.43×10^{-11} g of nucleic acids (which account for 47.45% to total dry mass) and 48.14×10^{-11} g (34.92%).

The results with respect to nucleic acids (DNA and RNA) are not unexpected since the ratio of DNA only to total dry mass determined by U.V. and interference microscopy has been shown to be 40% in living ram sperm heads (Davies *et al.* 1954).

The trypsin action may have not been complete, but since the time of treatment was the same for silk glands and Malpighian tubes, differences in the results may be analysed and considered of interest. Histones are in a higher percentage in the silk glands than in the Malpighian tubes perhaps due to a greater specialization for protein synthesis in the silk glands, while Malpighian tubes are specialized for excretion of lipid globules (Mello and Bozzo 1969). Therefore, in the silk glands whose genes are more directly activated, providing the protein synthesis, a large repression of other genes not concerned with this event would occur. Then histones in the silk glands are 4 times those of the Malpighian tubes. Histones act as inhibitors of RNA synthesis in the nuclei (Allfrey 1963) and are associated with those genes which are permanently inactive, since many genes must remain repressed throughout the whole life of a cell (Butler 1966).

The dry mass values in the Malpighian tubes of *Melipona quinquefasciata* are slightly higher than those of *Melipona quadrifasciata*, while the nuclear volumes are approximately the same in both species (Mello and Silveria 1970). Then one can assume that the differences in nuclear volumes are probably concerned with different degrees of hydration in the nuclei besides increase in acidic proteins. DNA cytophotometric data suggest that the nuclei in the larval Malpighian tubes of *M. quinquefasciata* undergo three DNA replications while in *M. quadrifasciata* they undergo four DNA replications (Mello and Silveira 1970). Micro-interferometric data support this interpretation and show that the same pattern occurs in the silk glands.

Summary

The dry masses of isolated polyploid nuclei from somatic tissues of bees may be established by micro-interferometric procedures and are often in accordance with cytophotometric values for DNA. Increases over those expected from

polyploidy in nuclear volumes and in nuclear dry masses of *Melipona quadrifasciata* Malpighian tubes may be due to non-histonic proteins, but in *Melipona quinquefasciata* such increases are also probably concerned with hydrating factors. The histone content removed by trypsin in the last larval instar of *Melipona quadrifasciata* is higher in the silk glands than in the Malpighian tubes, perhaps due to a large repression of genes not concerned with protein synthesis in the silk glands.

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