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Condensação cromatínica e metilação de DNA investigadas em abelhas *Melipona quadrifasciata* e *Melipona rufiventris* (Hymenoptera, Apoidea)

Tese apresentada ao Instituto de Biologia para obtenção do título de Doutor em Biologia Celular e Estrutural, na área de Biologia Celular.

Orientadora: Profª Drª Maria Luiza Silveira Mello

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Gênero Melipona: A) *Melipona quadrifasciata* (Mandaçaia). B) *Melipona rufiventris* (Urucu-amarela). http://www.abelhas.ufc.br/fotos.htm

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CONTEÚDO

RESUMO

O gênero *Melipona* (abelhas sem ferrão) tem sido dividido em dois grupos, com base no seu conteúdo em heterocromatina revelada com a técnica de banda-C em cromossomos mitóticos. *Melipona quadrifasciata* e *Melipona rufiventris* apresentam, respectivamente, níveis baixos e altos de heterocromatina. Na suposição de que cromatina condensada possa ser rica em seqüências de DNA metiladas, *M. quadrifasciata* e *M. rufiventris* poderiam então apresentar diferenças em conteúdo de seqüências CpG metiladas. Se isso acontecesse, as diferenças poderiam ser reveladas pela comparação de valores Feulgen-DNA obtidos por análise de imagem de células tratadas com as enzimas de restrição *Msp* I e *Hpa* II, que distinguem entre seqüências metiladas e não metiladas. *Msp* I e *Hpa* II clivam as seqüências –CCGG-, porém não há clivagem pela *Hpa* II se a citosina do dinucleotídeo central CG for metilada. Neste trabalho, túbulos de Malpighi de larva de último estádio de *M. quadrifasciata* e *M. rufiventris* submetidos à reação de Feulgen precedida pelo tratamento com *Msp* I e *Hpa* II tiveram suas células analisadas por microespectrofotometria de varredura automática. Para esse material houve necessidade do desenvolvimento prévio de um ajuste metodológico que tornasse a reação de Feulgen reveladora apenas de DNA, visto que ocorria reação plasmal; isto foi conseguido com um tratamento por boridreto de sódio a 5% e acetona/clorofórmio (1:1, v/v) antecedendo a reação de Feulgen. Também, embora a definição de altos e baixos conteúdos de heterocromatina em *Melipona* pela técnica de banda-C não fosse extensível à cromatina de núcleos interfásicos dos túbulos de Malpighi dessas abelhas, demonstrou-se que a depurinação do DNA em *M. quadrifasciata* era mais rápida do que a de *M. rufiventris*, confirmando, maiores teores de cromatina

condensada em *M. rufiventris*. Os valores Feulgen-DNA para a heterocromatina de *Melipona rufiventris* e para a pouca heterocromatina somada a alguns domínios de eucromatina de *Melipona quadrifasciata* diminuíram após tratamento com *Msp* I, porém ficaram inalterados após tratamento com *Hpa* II. Conclui-se que seqüências CpG metiladas podem estar contidas em diferentes compartimentos cromatínicos, conforme a espécie do gênero *Melipona* considerada, e que os seus efeitos silenciadores possam atuar induzindo uma mesma fisiologia celular.

ABSTRACT

The genus *Mellipona* has been divided into two groups based on its heterochromatin content revealed by C-banding pattern in mitotic chromosomes. *Melipona quadrifasciata* and *Melipona rufiventris* show low and high heterochromatin content, respectively. Supposing that condensed chromatin may be rich in DNA methylated sequences, *M. quadrifasciata* and *M. rufiventris* could, thus, show differences regarding their content of CpG methylated sequences. In this situation, such differences could be revealed by comparing the Feulgen-DNA values acquired after image analysis of cells treated with restriction enzymes *Msp* I and *Hpa* II, which distinguish between methylated and non-methylated sequences. *Msp* I and *Hpa* II break the CCGG sequences. Nevertheless, *Hpa* II is ubable to break the DNA strand if the cytosine from the central nucleotide pair CG is methylated. In this work, Malpighian tubules from larvae from the last stage of *M. quadrifasciata* and *M. rufiventris*, subjected to the Feulgen reaction after by treatment with *Msp* I and *Hpa* II, were analysed in automatic scanning microspectrophotometry. Since a plasmal reaction was observed in this material, it was previously necessary the development of a methodological adjustement to make the Feulgen reaction specific to DNA. This was achieved by treatment of material with 5% sodium borohydrade followed by acetone-chloroform (1:1, v/v) before the Feulgen reaction. Also, although the definition of high and low heterochromatin content in *Melipona* after C-banding technique is not applicable to the chromatin of interphasic nuclei in Malpighian tubules of bees, it was demonstrated that DNA depurination in *M. quadrifasciata* was faster than that of *M. rufiventris*, thus confirming that this species has a higher condensed chromatin content. The

Feulgen-DNA values for the heterochromatin of *Melipona rufiventris*, and for the heterochromatin besides some euchromatic domains of *Melipona quadrifasciata*, decreased after treatment with *Msp I*, remaining, however, unaltered after treatment with *Hpa II*. In conclusion, methylated CpG sequences may be part of different chromatin compartments, according to the considered species of the genus *Melipona*, and that their silencing effects may act by inducing the same cell physiology.

1 – INTRODUÇÃO

1.1- Abelhas do gênero *Melipona*

O gênero *Melipona* Illiger, 1806, consiste de um grupo de abelhas com ferrão atrofiado, distribuídas em grande parte das regiões de clima tropical e em algumas importantes regiões de clima temperado subtropical, estando presentes na maior parte do continente sul-americano (Camargo, 1989; Camargo & Pedro, 1992; Nogueira-Neto, 1997; Michener, 2000).

A análise citogenética do gênero *Melipona* foi iniciada por Kerr (1948) e continuada por diversos outros pesquisadores. Esses estudos revelaram para tal grupo o número cromossômico de 2n = 18 e 2n = 20 (Kerr, 1972; Tambasco et al., 1979; Rocha & Pompolo, 1998; Rocha et al., 2002) sendo mais comumente encontrado 2n = 18. Baseando-se em resultados obtidos com o uso da técnica de banda-C, que evidencia regiões heterocromáticas (Sumner, 1972), o gênero *Melipona* foi dividido em dois grupos: o primeiro (I) compreenderia espécies com baixo conteúdo de heterocromatina, incluindo *M. bicolor bicolor*, *M. quadrifasciata* (Mandaçaia), *M. marginata* e *M. asilvai*; o segundo grupo (II) compreenderia espécies com alto conteúdo de heterocromatina, incluindo *M. seminigra fuscopilosa*, *M. capixaba*, *M. scutellaris, M. captiosa* e *M. rufiventris* (Urucu-amarela) (Rocha & Pompolo, 1998; Rocha et al., 2002; Pompolo, 2002- comunicação pessoal). Esta classificação foi obtida para cromossomos mitóticos metafásicos de gânglios cerebrais (Rocha & Pompolo, 1998; Rocha et al., 2002; Pompolo, 2002- comunicação pessoal).

Nos cromossomos de espécies de *Melipona* com um alto conteúdo de heterocromatina, definida por resposta positiva à técnica de banda-C, a eucromatina aparece restrita à extremidade dos cromossomos (Rocha et al., 2002).

Os altos conteúdos de heterocromatina, que caracterizam as espécies do grupo II de *Melípona*, são interpretados como tendo evoluído através de amplificação de segmentos heterocromáticos, ou por adição de heterocromatina (Rocha et al., 2002).

1. 2 – Heterocromatina e eucromatina

Cromatina e cromossomos são constituídos por dois tipos de domínios: eucromatina e heterocromatina. Muito ainda se desconhece sobre a heterocromatina, embora suas propriedades básicas remontem a dezenas de anos atrás, estando entre elas a condensação permanente ao longo do ciclo celular, pobreza em genes, relativo silenciamento gênico, replicação mais lenta do DNA e, em algumas vezes, presença de DNA repetitivo (Heitz, 1935; Mello, 1978; Babu & Verma, 1987; Pieczarka & Mattevi, 1998; Wallrath, 1998; Craig, 2004). Segundo Grewal e Moazed (2003) os domínios de heterocromatina são geralmente inacessíveis aos fatores de ligação ao DNA e silentes em termos transcricionais. Os domínios de eucromatina, por outro lado, definem aquelas partes do genoma mais acessíveis e ativas em transcrição. Grandes blocos de heterocromatina estariam em estruturas cromossômicas como os centrômeros e telômeros, enquanto domínios heterocromáticos menores estariam mais espalhados ao longo dos cromossomos (Grewal & Elgin, 2002).

Proteínas específicas da heterocromatina estão associadas com DNA repetitivo da região dos centrômeros, sendo necessárias para que, por exemplo, a ligação das cromátides

irmãs e a segregação cromossômica ocorram de modo adequado (Bernard et al., 2001; Peters et al., 2001; Nonaka et al., 2002; Hall et al., 2003; Grewal & Moazed, 2003). A apresentação na forma de heterocromatina estabilizaria seqüências de DNA repetitivo nos centrômeros, telômeros e outras partes no genoma, impedindo recombinação entre repetições homólogas (Grewal & Klar, 1997; Guarente, 2000; Grewal & Moazed, 2003).

Além do seu papel em manter a estabilidade do genoma, a heterocromatina desempenha papel importante na regulação da expressão gênica durante o desenvolvimento e a diferenciação celular. O próprio empacotamento do DNA na heterocromatina (condensação cromatínica) exerceria controle epigenético sobre importantes processos biológicos. Isto acontece, por exemplo, na inativação de um dos cromossomos X de células somáticas de mamíferos placentários XX, permitindo um mecanismo de compensação de dose (Avner & Heard, 2001).

Algumas propriedades da heterocromatina a tornam adequada a processos que requerem inativação estável de estados de expressão por longos períodos. Assim, a heterocromatina é herdada epigeneticamente e de modo estável através de muitas divisões celulares, que podem acontecer sob diferentes condições do desenvolvimento e ambientais (Grewal & Moazed, 2003). Além disso, o mecanismo de montagem da heterocromatina e do espalhamento da heterocromatina, a partir de sítios de nucleação para regiões vizinhas do DNA, permitiria uma transição de controle genético seqüência-específico para um controle epigenético independente de seqüência do DNA (Grewal e Moazed, 2003).

Muitos dos fatores *trans-acting* requeridos para montagem da heterocromatina são tanto enzimas que modificam diretamente as histonas H3 e H4, como fatores que se ligam a elas. Entre essas proteínas podem ser citadas as de complexos SIR, que contêm entre seus

componentes deacetilases de histona, encontradas em leveduras e metazoários (Shankaranarayana et al., 2003) e proteínas HP1, que se ligam especificamente a caudas de histona H3 metiladas na lisina 9, encontradas em seres humanos, camundongos e em *Drosophila* (Aagaard et al., 1999; Grewal e Moazed, 2003; Gilbert et al., 2005).

Admite-se também que a metilação do DNA contribua para a manutenção dos sítios de cromatina silenciada nos eucariotos superiores com genomas complexos (Grewal e Moazed, 2003). Parece existir um mecanismo de retroação entre metilação do DNA e de histonas, de modo que um promova a manutenção do outro (Tamaru & Selker, 2002; Johnson et al., 2002; Soppe et al., 2002), orquestrando assim a manutenção de um estado silenciado da cromatina (Grewal e Moazed, 2003; Lehnertz et al., 2003). A demonstração de que a manutenção da heterocromatina é influenciada pela metilação do DNA reside no fato de que o tratamento de células pela 5-azacitidina resulta na sua descondensação (Gilbert et al., 2005).

Ao longo da evolução, a heterocromatina aparece em paralelo com o aumento em tamanho dos genomas eucarióticos (Wallrath, 1998; Grewal & Elgin, 2002). Segundo Avramova (2002) as regiões densamente compactadas ou a formação da estrutura altamente ordenada da cromatina teria sido vantajoso por estar associado ao controle de excesso de DNA predominantemente estranho, como transposons e retrovírus. A invasão de elementos genéticos móveis seria deletéria à integridade do genoma, e possivelmente por isso, as células desenvolveram diferentes estratégias para silenciá-los, segregando-os em compartimentos pobres em genes denominados de heterocromatina (Avramova, 2002).

1.3 - A metilação do DNA e ilhas 5´-CpG-3´

A metilação do DNA é uma modificação epigenética reversível bem definida, constatada na maioria dos genomas dos organismos (Singer & Berg, 1991; Bestor, 2000). Refere-se à transferência de um grupo metil (CH_3) da S-adenosil metionina das metilases ao carbono da posição 5 do anel cíclico da citosina, formando a 5-metilcitosina (^{5}mC) ou ao resíduo do nitrogênio não cíclico da adenina específica formando N^6-metiladenina (Singer & Berg, 1991; Pradhan & Esteve, 2003; Issa, 2005).

Os resíduos de 5-metilcitosuna (^{5}mC) são encontrados principalmente em dinucleotídeos palindrômicos 5´-CpG-3´ e trinucleotídeos 5´-CpNpGp-3´ simétricos (N=C, A, T), este último mais comum e predominante no genoma de plantas superiores. Com baixa freqüência em seqüências não simétricas a ^{5}mC pode ser encontrada com os resíduos de timina formando ^{5}mCpT, com adenina formando ^{5}mCpA e com citosina formando ^{5}mCpC (Van der Ploeg & Flavell, 1980; Gruenbaum et al., 1981; Bird, 1986; Singer & Berg, 1991; Siegfried & Cedar, 1997; Finnegan et al., 1998; Costello & Plass, 2001).

Os genomas eucarióticos não são metilados uniformemente, contendo regiões metiladas intercaladas com regiões não metiladas. Regiões curtas de DNA caracterizadas por níveis de CG mais altos do que a média do DNA são chamadas de ilhas 5´-CpG-3´; estendem-se de 0,5kb a 5kb e ocorrem em média a cada 100kb (Kundu & Rao, 1999; Singal & Ginder, 1999; Das & Singal, 2004). Aproximadamente 60 a 90% dos dinucleotídeos CpG são metilados nos genomas dos mamíferos (Kundu & Rao, 1999) localizadas nas extremidades 5´ dos genes e nas seqüências intergênicas repetidas (The International Human genome sequencing consortium, 2001; Garcia-Maneiro et al., 2002; Takai & Jones,

2003). No genoma humano, há uma variação substancial em conteúdo de CG associado a fragmentos extensos. Regiões ricas em CG e pobres em CG podem ter diferentes propriedades biológicas como a densidade de genes, a composição de seqüências repetidas, a correspondência com bandas citogenéticas e a proporção de recombinação (The International Human genome sequencing consortium, 2001). As seqüências 5´-CpG-3´ metiladas contêm freqüentes sítios de restrição à ação de *Hpa* II e, conseqüentemente, podem ser detectadas como tênues fragmentos de *Hpa* II ou HTF (*Hpa* II tiny fragment) (Kundu & Roa, 1999).

1.4 - A metilação dos genomas

A metilação do DNA genômico tem sido estudada extensivamente e encontrada em bactérias (Singer & Berg, 1991), em cianobactéria *Nostoc commune* (Jager & Potts, 1988) na maioria dos grandes grupos filogenéticos de eucariotos, como nas espécies analisadas em algas verdes *Chlamydomonas reinhardi*, *Euglena gracilis* (Hattman et al., 1979), em fungos *Neurospora crasssa* (Selker et al., 2003*), Candida albicans* (Russel et al., 1987), *Ascobolus immersus* (Goyon et al., 1996), *Saccharomyces cerevisae* (Hattman et al., 1979) [embora confirmado ausente nos resultados obtidos por Proffitt et al., (1984)], nos protozoários *Paramecium aurelia*, *Paramecium candatum*, *Tetrahymena pyriformis*, *Colpoda inflanta* (Gorovsky et al., 1973; Cumings et al., 1974; Gutierrez et al., 2000), *Trypanosoma cruzi* (Rojas & Galanti, 1990), em Porifera (ausente em algumas espécies), Cnidária, Nemertea (altos níveis), Mollusca, Annelida, Crustacea, alguns grupos de insetos, Bryozoa, Equinodermata (Regev et al., 1998), em todos os vertebrados (Tweedie et al., 1997) e plantas (Finnegan et al., 1998). É relatado como ausente em alguns fungos como

em *Dictiostelium discoideum* (Smith & Ratner, 1991), alguns platelmintos *Schistosoma mansoni* (Fantappié et al., 2001), e totalmente ausente no nematóide *Caenorhabditis elegans* (Simpson et al., 1986).

Dados analisados por Urieli-Shoval et al. (1982) para embriões, pupas, larvas e adultos de *Drosophila melanogaster* não detectaram resíduos de citosina metilados, o que os levou a concluir que, em contraste com a maioria dos eucariotos, *Drosophila melanogaster* e talvez outros insetos, fossem isentos de m^5C em seu DNA. Apesar deste relato, metilação de DNA foi constatada em cromossomos politênicos nas espécies *Sciara coprophila*, *Drosophila melanogaster* e *Drosophila persimilis* (Eastman et al., 1980; Achwal et al., 1983). Segundo Lyko et al. (2000) alguma metilação ocorreria em *Drosophila*, porém restrita a fases iniciais do desenvolvimento embrionário, com os embriões apresentando 0,3% de CpG metiladas. Gowher et al. (2000), por outro lado, relatam que a metilação do DNA em *Drosophila* é detectável em todos os estágios do seu desenvolvimento. A 5-metilcitosina ocorreria no DNA de *Drosophila melanogaster* ao nível de aproximadamente 1 em 1000-2000 resíduos de citosina no adulto.

A maioria dos genomas dos invertebrados apresentam níveis moderados de seqüências CpG metiladas concentrados em pequenos segmentos de DNA metilado separados por segmentos equivalentes não metilados (Bird, 2002).

1.5- A importância da metilação do DNA

Desde 1925 se sabe que as bactérias contêm a 5-metilcitosina, mas somente nos anos 40 e 50 foi confirmada a sua presença em animais e plantas (Kuzmin & Geil, 2001),

gerando interesse e contradições para entender o seu significado nos genomas (Singal & Ginder, 1999). Em procariotos, a metilação do DNA desempenha importante função na proteção dele próprio. O sistema de restrição-modificação é composto de DNA metiltransferase e endonuclease de restrição, em que ambas reconhecem a mesma seqüência alvo no DNA, porém, a DNA metiltransferase (DNMT) protege o DNA da clivagem por endonucleases de restrição graças a metilações introduzidas (Singer & Berg, 1991; Finnegam & Kovac, 2000).

Em plantas a metilação é essencial para o desenvolvimento. Em *Arabidopsis* e em outras plantas, níveis reduzidos de metilação do DNA acham-se associados a extensas anomalias como a perda da dominância apical, redução da estatura, forma e tamanho das folhas alteradas, comprimento reduzido da raiz e redução da fertilidade (Finnegan et al., 1998).

Nos vertebrados, como a metilação do DNA é um aspecto comum (Tweedie et al., 1997**),** a mutação de ambos os alelos do gene para DNA metiltransferase (DNMT1) é letal ao desenvolvimento embrionário, conforme constatada em camundongos (Li et al., 1992). A metilação do DNA é também um fenômeno que atua na saúde humana. Mutação no gene para enzima DNMT3b, responsável pela metilação *de novo* nas regiões heterocromáticas adjacentes ao centrômero nos cromossomos 1, 9 e 16, causa a síndrome imunodeficiente da instabilidade da região centromérica e anomalia facial (ICF) (Hansen et al., 1999; Xu et al., 1999; Ehrlich et al., 2001a; Ehrlich et al., 2001b; Tsuda al., 2002). Há também relatos de que distúrbio no padrão de metilação do DNA induzam a gênese e evolução clínica de câncer (Kwong et al., 2002; Uehara et al., 2003; Matsushita et al., 2004), esquizofrenia (Costa et. al., 2002; Sharma, 2005) e diabetes tipo 2 (Maier & Olek, 2002).

Algumas funções-chaves desempenhadas pela metilação do DNA são: a manutenção da estabilidade do genoma, por silenciar a expressão de elementos genéticos transponíveis de conseqüências tóxicas para a célula (LINEs, SINEs, Alu, LTR e transposons de DNA) (Lewin, 2001; The International Human genome sequencing consortium, 2001), promoção da estabilidade genética de seqüências repetidas (CGG)n, (CTG)n e dinucleotideos (CpG)n (Nichol & Pearson, 2002); participação no desenvolvimento e na diferenciação celular (Monk et al., 1987); controle da expressão gênica tecido-específico (Jones, 1999); participação na inativação do cromossomo X de fêmeas de mamíferos placentários (Mohandas et al., 1981), organização da cromatina e *imprinting* genômico (Cerda & Weitzman, 1997), e transição do DNA da forma B para Z (Liang & Jasin, 1995). Alterações no padrão de metilação conferem instabilidade a certas regiões genômicas, por alterar a estrutura da cromatina, o que favoreceria a instalação de deleções, inversões e perda de cromossomos. A metilação do DNA também pode estar envolvida na prevenção da recombinação de seqüências repetitivas quanto na formação da cromatina inativa (heterocromatina) (Kokalj-Vokac et al., 1993; Liang & Jasin, 1995; Pogribny et al., 1995; Grewal & Moazed, 2003; Frigola et al., 2005).

1.6- Bandamento cromossômico e heterocromatina: banda-C

O bandamento cromossômico resulta de uma variação nas propriedades de coloração ao longo dos cromossomos, revelando certos níveis de organização cromatínica de valor prático na identificação de cromossomos ou parte deles (Sumner, 1990). Uma das técnicas de bandamento cromossômico, a banda-C, induz um padrão diferenciado ao longo dos braços cromossômicos ou do cromossomo inteiro, evidenciando regiões menos coradas

e mais coradas chamadas de bandas. Os padrões de bandas são específicos para a espécie (Babu & Verma, 1987; Sumner, 1990).

A técnica de banda-C se baseia na remoção preferencial do DNA contido nas regiões eucromáticas, retendo-se grande quantidade de DNA nas regiões heterocromáticas. Os processos químicos responsáveis pela perda do DNA durante a técnica de banda-C atuam segundo três etapas com funções específicas: tratamento ácido, tratamento alcalino e tratamento com solução salina. O tratamento ácido com 0,2 HCl N seguido de tratamento alcalino por Ba $(OH)_2$ depurina o DNA e causa a ruptura de parte dos sítios apurínicos, bem como desnaturação irreversível do DNA. A extração do DNA depurinado ocorre durante a etapa final de incubação com solução salina, resultando na β-eliminação de sítios depurinados (pequenos fragmentos de DNA que são facilmente liberados dos cromossomos), permanecendo DNA não rompido (Sumner, 1990).

A coloração seletiva das regiões eucromáticas e heterocromáticas nos cromossomos facilitam a montagem do cariótipo, a detecção de heteromorfismos e rearranjos cromossômicos ocorridos durante a evolução (Imai et al., 1988, 1994; Sumner, 1990; Imai, 1991).

1.7 - Reação de Feulgen e heterocromatina

A reação de Feulgen foi introduzida por Feulgen e Rossenbeck, como específica para DNA (Mello & Vidal, 1978; Mello, 1997). Esta reação compreende duas etapas: 1- hidrólise ácida com HCl, com o objetivo de desnaturar o DNA e remover deste as purinas, obtendo-se, assim, um ácido apurínico; 2– tratamento com o reativo de Schiff, com alta afinidade por grupamentos aldeídicos, gerados, no caso, ao nível de desoxirribofuranoses,

quando da remoção das purinas. A ligação das moléculas do reativo de Schiff ao ácido apurínico confere a este uma cor magenta característica. O uso de reação de Feulgen para o DNA resultou em um grande progresso para o conhecimento sobre a distribuição deste componente em vários tipos de células bem como possibilitou a sua quantificação, pois demonstrou-se ser ela de caráter estequiométrico, ou seja, a sua resposta (intensidade da cor) é proporcional à concentração do DNA. Desta forma, a reação de Feulgen é o procedimento citoquímico mais conhecido e utilizado para as determinações quantitativas convencionais de DNA ao nível de células e tecidos.

Graças à quantificação da resposta à reação de Feulgen, estabeleceu-se que o conteúdo médio de DNA por lote cromossômico é no geral constante para uma mesma espécie, animal ou vegetal, e que a replicação do DNA ocorre na interfase (fase S). Também possibilitou a detecção de poliploidia em núcleos interfásicos de diversos órgãos, como no caso do fígado e do coração, em vertebrados, e da maioria dos insetos, inclusive de abelhas (Mello & Takahashi, 1969, 1971; Mello, 1970). Permitiu, por outro lado, que se determinasse a ocorrência de aneuploidias e poliploidias em vários tipos de tumores e infecções por vírus de DNA (Mello & Vidal, 1978).

No que se refere às abelhas, foram constatados altos níveis de ploidia somática em órgãos como túbulos de Malpighi e glândulas salivares em espécies do gênero *Melipona* (Mello & Takahashi, 1969; Mello & Silveira, 1970; Mello et al., 1970) bem como em *Apis mellifera* (Mello & Takahashi, 1971) através de quantificação de valores Feulgen-DNA. Particularmente em *Apis mellifera* constatou-se que os conteúdos de DNA dos zangões eram maiores do que os das operárias, porém menores do que das rainhas, sendo tais

diferenças relacionadas à biomassa das diferentes castas e seus requerimentos funcionais específicos (Mello & Takahashi, 1971).

A quantificação de valores Feulgen-DNA nas células é realizada com o uso de analisadores de imagem por sistema de vídeo ou por microespectrofotometria. A microespectofotometria de varredura automática, controlada por computador é considerada um dos procedimentos mais precisos na avaliação dos conteúdos Feulgen-DNA (Mendelsohn, 1966; Hale, 1966), e que envolve a determinação de absorbâncias (A) e áreas absorventes (S), sendo o processo efetuado ponto por ponto ao longo do trajeto varrido. Após sua finalização, os valores AxS totais são integralizados, obtendo-se valores Feulgen-DNA em unidades arbitrárias. Estas unidades poderão vir a ser convertidas em valores absolutos (picogramas) se houver necessidade de comparação a controles específicos, com conteúdos de DNA previamente estabelecidos. Na prática, em se tratando de comparações entre ensaios experimentais e controles, geralmente se trabalha com dados em unidades arbitrárias.

Durante a hidrólise ácida da reação de Feulgen o número de aldeídos produzidos com a remoção das purinas do DNA aumenta com o avanço do tempo, alcança um máximo e depois se reduz. Isto é conhecido como cinética de hidrólise de Feulgen, onde se identificam as fases de avanço na depurinação do DNA (porção ascendente de uma curva de hidrólise) até atingir o máximo (platô da curva de hidrólise), seguindo-se solubilização do ácido apurínico (porção descendente da curva de hidrólise) (Mello & Vidal, 1980; Mello, 1997). Para a avaliação de conteúdos Feulgen-DNA se escolhe um tempo de hidrólise que corresponda ao platô da curva de hidrólise.

O padrão de uma curva de hidrólise é típico para um tipo de cromatina de determinada espécie, alterando-se com a diferenciação, o desenvolvimento, a fisiologia nuclear e celular, e durante o envelhecimento (Mello & Vidal, 1980). Quando se analisam perfis de curvas de hidrólise de Feulgen para heterocromatina e eucromatina em separado são nítidas as diferenças (Mello, 1979, 1983). De modo geral, a cinética de hidrólise de Feulgen é afetada pela supra-organização cromatínica, sendo que a depurinação do DNA e/ou a solubilização do ácido apurínico se torna mais lenta em núcleos com cromatina mais condensadas e mais aceleradas em núcleos com cromatina mais frouxa (Mello, 1979; Mello & Vidal, 1980; Miyamoto et al., 2005).

1.8- Enzimas de restrição e pesquisa de DNA com seqüências CG metiladas

As nucleases se constituem em uma ferramenta específica para a manipulação do DNA e do RNA. Cada enzima pode ser descrita de acordo com sua especificidade e a reação catalisada. Há enzimas de restrição que clivam somente o DNA; outras, como a RNAse pancreática, hidrolisam somente o RNA. Há outras enzimas que atuam sobre ambos. As nucleases são divididas em endonucleases e exonucleases (Singer & Berg, 1991). A exonuclease requer para iniciar a clivagem do substrato que este contenha um polinucleotídeo na sua extremidade ou próximo ao término da sua cadeia (Singer & Berg, 1991). As endonucleases ou enzimas de restrição podem hidrolisar moléculas circulares, clivando sempre as ligações fosfodiéster internas e produzindo polinucleotídeos de diferentes tamanhos. A evolução manteve diferentes espécies de bactérias com uma única

endonuclease que lhes permitiu distinguir o seu próprio DNA do DNA estranho, de modo que a própria natureza forneceu aos cientistas um conjunto de reagentes específicos para dissecar o DNA (Singer & Berg, 1991). Apesar da existência de muitas diferentes endonucleases, cada uma reconhece uma seqüência específica de nucleotídeos na molécula do DNA (Singer & Berg, 1991).

As endonucleases que reconhecem a mesma seqüência de nucleotídeos no DNA e que são derivadas de diferentes organismos são chamadas de isoesquizômeros (Sambrook et al. 1989; Hoenecke, 1994). As enzimas *Hpa* II (derivada de *Haemophilus parainfluenzae*) e *Msp* I (derivada da *Moraxella sp.*), reconhecem a mesma seqüência de tetranucleotídeos no DNA (5´-CCGG-3´), mas a *Hpa* II corta a sequência não metilada, enquanto a *Msp* I corta tanto a sequência metilada na citosina interna (5´-C^{5m}CGG-3´) como a não metilada (5´-CCGG-3´) (www.sigma-aldrich.com; www.thelabrat.com/restriction; Sambrook et al., 1989; Singer & Berg, 1991; Hoenecke, 1994; Xu et al., 2000; Hou et al., 2004).

Quando a C externa na seqüência CCGG é metilada, tanto a *Msp* I como a *Hpa* II não podem clivá-la (www.neb.com/nebecomm/products/productR0106.asp).

As enzimas de restrição fornecem um meio para quebrar genomas complexos ou segmentos longos de DNA em pequenas unidades reprodutíveis. As pequenas unidades podem ser separadas umas da outras baseando-se nos seus diferentes tamanhos (Singer & Berg, 1991).

A ocorrência de metilação na C interna de seqüências –CCGG- no DNA de cromossomos e de cromatina condensada de núcleos interfásicos pode ser avaliada *in situ*, quando se tratam os preparados cromossômicos e cromatínicos com as enzimas de restrição

Msp I e *Hpa* II (Mezanotte et al., 1983; Bianchi et al., 1986; Sentis et al., 1994; Gonsálves et al., 1995; Mello et al., 2000). Quando o tratamento enzimático é seguido por reação de Feulgen é possível se avaliar se a resposta promovida pelas duas enzimas afeta valores Feulgen-DNA obtidos em controles e assim se concluir pela presença de –CCGG- bem como de –C^{5m}CGG- nos materiais pesquisados (Mello et al., 2000). Com esse procedimento pôde ser determinado, por exemplo, que o grau de metilação da C interna de seqüências –CCGG- não influencia a remodelação cromatínica que se segue à transformação de células fibroblásticas de camundongo NIH3T3 com o oncogene c-Ha-*ras* (Mello et al., 2000).

2 – JUSTIFICATIVA

As abelhas sem ferrão *Melipona rufiventris* (Uruçu-amarela) e *Melipona quadrifasciata* (Mandaçaia) pertencem a grupos com alto e baixo conteúdo de heterocromatina, respectivamente, definidos em termos de resposta à técnica de banda-C em cromossomos mitóticos de gânglios cerebrais larvais (Rocha & Pompolo, 1998; Rocha et al., 2002; Pompolo, 2002- comunicação pessoal). Já foi relatado que DNA rico em CG ocorre em espécies de *Melipona* com alto e baixo conteúdo de heterocromatina (Rocha et al., 2002). Porém, não se sabe ainda se na cromatina de núcleos interfásicos os sítios CpG poderiam se encontrar ao menos em parte metiladas nem se, no caso disso ocorrer, haveria alguma correlação com os níveis de condensação cromatínica admitidos como diferentes para essas abelhas.

A pesquisa de presença de seqüências de DNA ricas em CG e de metilação na C interna das seqüências –CCGG- é factível em núcleos somáticos inteiros tratados com as enzimas de restrição *Msp* I e *Hpa* II e, em seguida, coradas pela reação de Feulgen (Mello et al., 2000). Tanto *Msp* I quanto *Hpa* II clivam a sequência –CCGG-, mas *Hpa* II não cliva tal seqüência, se a C interna estiver metilada. Preparados corados com a reação de Feulgen, por outro lado, podem ter seu DNA revelado e quantificado por microespectofotometria de varredura. Assim, é possível investigar a presença de seqüências CpG totais e metiladas na cromatina total, ou selecionada quanto a seus níveis de condensação, em núcleos interfásicos de abelhas com diferente conteúdo de heterocromatina. Deste modo, estaria-se contribuindo com dados que visam esclarecer a relação entre cromatina condensada e metilação de DNA num grupo de insetos ainda pouco explorado quanto a este aspecto.

3 - OBJETIVOS

Segundo relatos prévios, abelhas das espécies *Melipona quadrifasciata* e *Melipona rufiventris* diferem em conteúdo de heterocromatina, definido com base na resposta à técnica de banda-C para cromossomos mitóticos. O presente trabalho tem como objetivo investigar em núcleos de células somáticas como as de túbulos de Malpighi, se a ocorrência diferencial de heterocromatina viria acompanhada por presença de sequências –CCGG- metiladas na citosina interna de seu DNA (artigo 3). Para tal, os preparados deveriam ser tratados por enzimas de restrição específicas, seguindo-se reação de Feulgen e quantificação de respostas Feulgen-DNA.

Uma vez que a resposta à reação de Feulgen nesses materiais revelou-se prejudicada por uma positividade no citoplasma conhecida como reação plasmal, houve necessidade do desenvolvimento de um ajuste metodológico que tornasse a reação de Feulgen reveladora apenas do DNA (artigo 1).

Tendo em vista também que a cromatina dos núcleos interfásicos das células epiteliais dos túbulos de Malpighi não revelou uma resposta à técnica de banda-C compatível aos achados em cromossomos mitóticos, houve necessidade de se estudar uma outra propriedade dessa cromatina: a resposta à reação de Feulgen. Esta poderia revelar diferenças em condensação cromatínica compatíveis aos relatos para cromossomos com outra metodologia (artigo 2).

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5 –ARTIGOS

1º Artigo

1. Mampumbu AR., Vidal BC. & Mello MLS. (2004). Feulgen staining in Malpighian tubules of meliponid bees: a methodological contribution. *Braz. J. Morph. Sci., 21*: 31.

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FEULGEN STAINING IN MALPIGHIAN TUBULES OF MELIPONID BEES: A METHODOLOGICAL CONTRIBUTION*

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ABSTRACT

Whole-mounted Malpighian tubules of larvae from two meliponid bee species fixed in acetic acid-ethanol showed a positive cytoplasmic staining with Schiff reagent when submitted to the Feulgen reaction in which acid hydrolysis was done in 4 M HCl at room temperature. The ability of various treatments applied before the Feulgen acid hydrolysis to abolish this cytoplasmic staining was examined. The aldehyde groups of phospholipids present in the cytoplasm of the Malpighian tubules were blocked or removed by sequential treatment with 5% sodium borohydride and acetone- chloroform (1:1, v/v) for 15 min each prior to HCl hydrolysis. This treatment is recommended in order to abolish the cytoplasmic (plasmal) reaction and to allow the reliable quantification of DNA by the Feulgen reaction and the discrimination of nuclear phenotypes in the Malpighian tubules of meliponid bees.

Key words: Aldehydes, cytoplasmic phospholipids, Feulgen staining, Malpighian tubules, meliponid bees, plasmal reaction

INTRODUCTION

As in most insects with complete metamorphosis, the larval organs in bees grow by endopolyploidy rather than by cell division [4]. The degrees of ploidy in cells of the larval Malpighian tubules of some bee species has been estimated in cytological preparations submitted to the Feulgen reaction in which the acid hydrolysis step was done in 1 M HCl at 60°C [3]. Currently, the hydrolytic step of the Feulgen reaction is frequently done in 4 M HCl at room temperature, mainly because of operational facilities and more reliable results obtained [5]. Under these conditions, when analyzing the Feulgen response in Malpighian tubule cells from bees of the genus *Melipona* fixed in absolute ethanol-glacial acetic acid (3:1, v/v) for 1 min, we have also observed a deep, positive staining in the cytoplasm of these cells. This additional staining, which is typical of cytoplasmic phospholipid aldehydes (plasmalogens) that react with Schiff reagent [7], can hinder the reliable quantification of nuclear DNA and the discrimination of nuclear phenotypes by microspectrophotometry or video image analysis.

In this study, we examined the ability of various treatments used before the Feulgen acid hydrolysis step to block or destroy the cytoplasmic aldehydes responsible for the strong reaction, while retaining the positive Feulgen staining of nuclear DNA.

Fifth instar predefecating larvae from *Melipona quadrifasciata anthidioides* Lep. and *Melipona rufiventris* Lep. (Hymenoptera, Apoidea) reared and supplied by the Federal University of Viçosa were used. Whole-mounted preparations of Malpighian tubules removed from the larvae in Ringer solution were fixed in absolute ethanol-glacial acetic acid (3:1, v/v) for 1 min, followed by rinsing in 70% ethanol for 1-5 min. The tubules were subjected to the Feulgen reaction, with hydrolysis in 4 M HCl at 25°C for 90 min followed by treatment with Schiff reagent for 40 min. The preparations were then rinsed three times in sulfurous water and once in distilled water before being air dried. The organs were cleared in xylene and mounted in natural Canada balsam (Vetec, Rio de Janeiro).

To define the cytoplasmic staining as a plasmal reaction [2,7], some fixed preparations were simply treated with Schiffreagent without the acid hydrolysis step. The abolishment of the cytoplasmic reaction was examined by varying the duration of the sulfurous water rinses from 3 to 10 min each and by treating the preparations with (a) 5% sodium borohydride solution [2], pH 9.8, for 1 to 15 min, (b) acetone-chloroform (1:1, v/v) for 15 min, or 5% sodium borohydride solution followed by acetone-chloroform (1:1, v/v) for 15 min each, prior to acid hydrolysis.

The preparations were observed and photographed in a Zeiss Axiophot II microscope (Oberkochen, Germany).

RESULTS

The tubules treated with Schiff reagent without prior acid hydrolysis showed the cytoplasmic staining typical of plasmal reaction (2,7).

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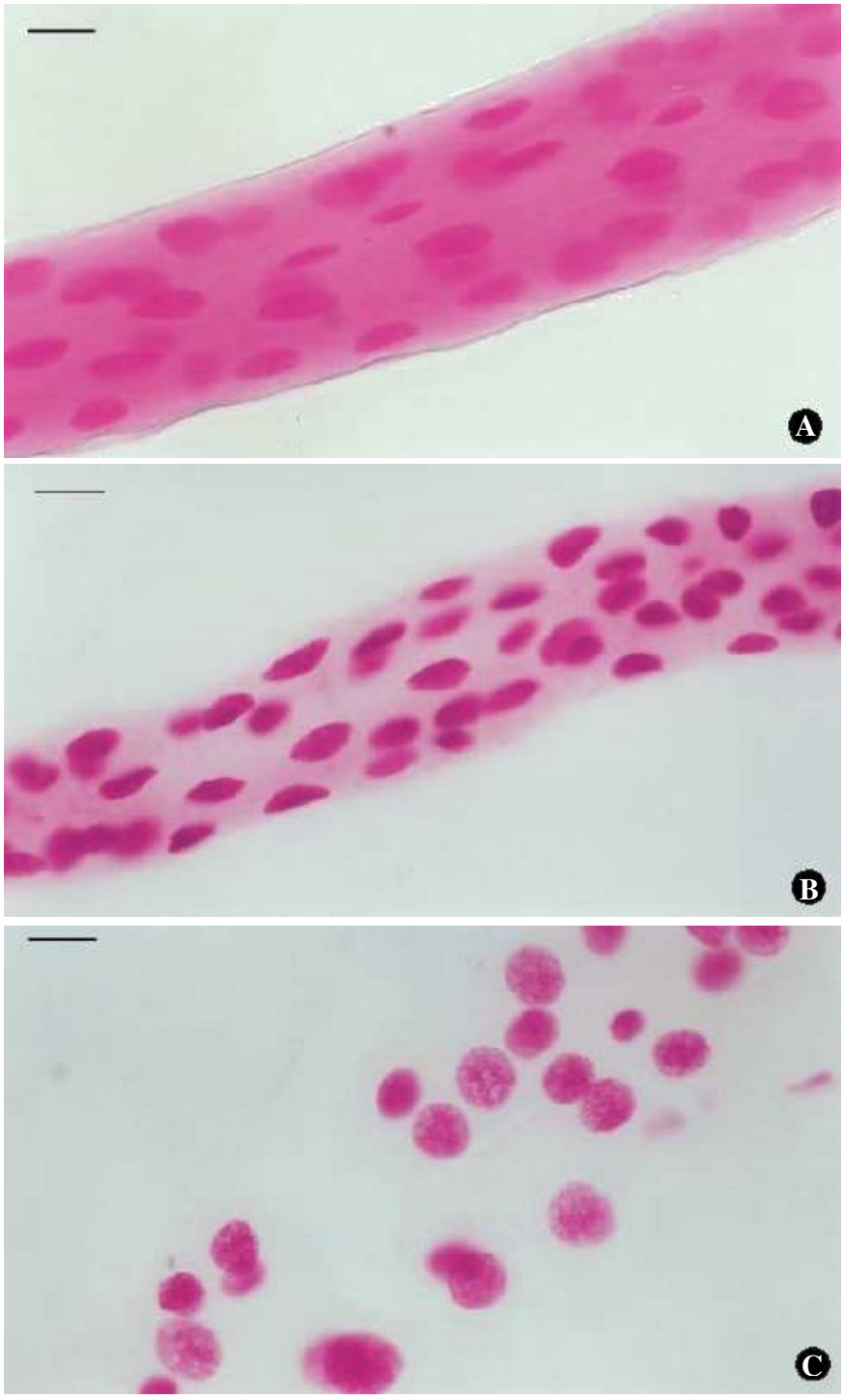

Figure 1. Feulgen-stained Malpighian tubules of *M. quadrifasciata*. **A** – Sulfurous water rinses lasting 10 min each. **B** – Reaction preceded by sodium borohydride treatment. **C** – Reaction preceded by treatment with sodium borohydride and acetone-chloroform. Bar = 30 ∝m.

Prolongation of the sulfurous water rinses up to 10 min each did not affect the positive cytoplasmic reaction in the Malpighian tubules of *M. quadrifasciata* submitted to the Feulgen reaction (Fig. 1A). Of the various treatments used prior to acid hydrolysis, that with borohydride solution decreased the intensity of the plasmal reaction after a 15 min exposure to this reducing agent (Fig. 1B), whereas the acetone-chloroform treatment did not affect the cytoplasmic reaction. In contrast, a combination of both treatments (sodium borohydride followed by acetone-chloroform for 15 min each) while using sulfurous water rinses lasting 3 min each after the Schiff reagent treatment totally abolished the plasmal reaction (Fig.1C). Similar results were obtained for *M. rufiventris.*

DISCUSSION

Our results indicate that aldehyde groups were responsible for the cytoplasmic staining seen in the Malpighian tubule cells of *M. quadrifasciata* and *M. rufiventris* after the Feulgen reaction. Alkaline solution of sodium borohydride reduces aldehydes to primary alcohols, [$4RCHO + NaBN_4 + 4H_2O \rightarrow 4RCH_2OH + B(OH)_3 + Na^+ + OH^-$] thus blocking the positive Schiff staining caused by aldehydes present in the tissue [2]. The complete removal of aldehydes present in the cytoplasm of Malpighian tubules required sodium borohydride treatment followed by acetone-chloroform (1:1, v/v). This observation indicated that the aldehydes involved were providedby phospholipids [2], a conclusion in agreement with the cytochemical demonstration of these components in the Malpighian tubules of *M. quadrifasciata* [6]. The use of whole-mounted preparations and of acid hydrolysis done at room temperature were probably responsible for preservation of the cytoplasmic aldehydes which reacted with the Schiff reagent. Similar reactions have been reported for frozen sections of other materials, but rarely in paraffin sections [7]. In a previous study of the DNA content of bee Malpighian tubules, no cytoplasmic staining was observed, probably because the Feulgen acid hydrolysis step had been done at 60ºC [3]. In bee organs that do not contain phospholipid granules such as those present in Malpighian tubules, no cytoplasmic staining is seen after the Feulgen reaction, even when the acid hydrolysis step is done at room temperature [1].

In conclusion, our results show that the potential interference by cytoplasmic aldehydes in the Feulgen reaction with acid hydrolysis at room temperature used in whole-mounted Malpighian tubules of meliponid bees can be eliminated by the sequential treatment with 5% sodium borohydride and acetone-chloroform (1:1, v/v) for 15 min each prior to the HCl hydrolysis.

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2º Artigo

2. Mampumbu AR. & Mello MLS. Chromatin condensation in interphase nuclei of the Malpighian tubules of *Melipana rufiventris* and *Melipona quadrifasciata* (Hymenoptera, Apoidea). Submetido à publicação.

Chromatin condensation in interphase nuclei of the Malpighian tubules of *Melipona rufiventris* and *Melipona quadrifasciata* (Hymenoptera, Apoidea)

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Abbreviated title: DNA content and chromatin condensation in *Melipona*

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Abstract - *Melipona quadrifasciata* and *M. rufiventris* are stingless bee species which present low and high heterochromatin content, respectively, on their mitotic chromosomes as assessed visually after the C-banding assay. However, these species do not show differences in the C-banding responses of their Malpighian tubule interphase nuclei. In the present study, the Feulgen-DNA response, which could inform on differences in DNA depurination due to differences in chromatin condensation was compared in the somatic cell nuclei of the Malpighian tubules of these species. It was hypothesized that differences in acid hydrolysis kinetics patterns, as assessed by the Feulgen reaction and studied microspectrophotometrically, could discriminate *M. quadrifasciata* and *M. rufiventris* interphase nuclei not distinguishable with the C-banding method. Feulgen-DNA values corresponding to more than one ploidy class were found in both species; these values at the hydrolysis time corresponding to maximal DNA depurination for each ploidy degree were higher in *M. quadrifasciata,* reflecting a higher DNA content in Malpighian tubule cell nuclei of this species compared to those of *M. rufiventris* at the same larval instar. The maximal Feulgen-DNA values of *M. quadrifasciata* after short (50 min) and long (90 min) hydrolysis times were found to be closer to each other, while those of *M. rufiventris* occurred sharply at the long hydrolysis time, indicating that DNA depurination in *M. quadrifasciata* occurred faster. This was probably related to differences in chromatin condensation being involved, and agreed with the idea that *M. rufiventris* contained more heterochromatin than *M. quadrifasciata*, which was supported by the analysis of results obtained with the parameter average absorption ratio. The depurination kinetics studied here with the Feulgen reaction revealed to be more pertinent than the C-banding technique to establish differences in levels of chromatin condensation for these cell nuclei.

Key words: Chromatin condensation; DNA content; Heterochromatin; Malpighian tubules; Stingless bees

INTRODUCTION

The genus *Melipona* Illiger, 1806, consists of a group of stingless bees with a wide distribution in the Neotropics (MICHENER 2000). Cytogenetic studies of these bees have shown chromosome numbers of $2n = 18$ and $2n = 20$ (KERR 1972; TAMBASCO *et al*. 1979; ROCHA and POMPOLO 1998; ROCHA *et al*. 2002), with the most common number being $2n = 18$.

Based on the relative amount of heterochromatin revealed by C-banding of mitotic chromosomes, the genus *Melipona* has been divided into two groups, one including species with low levels of heterochromatin, and the other containing species with high levels of heterochromatin (ROCHA and POMPOLO 1998; POMPOLO 2002 – unpublished data; ROCHA *et al*. 2002). Among several *Melipona* species with the same number of chromosomes (2n = 18) and studied with the C-banding technique, *M. quadrifasciata* has been classified as containing a low heterochromatin content (ROCHA and POMPOLO 1998; ROCHA *et al*. 2002), and *M. rufiventris* as presenting a high heterochromatin content (POMPOLO 2002 – personal communication). However, although we have confirmed POMPOLO'S (2002 – personal communication) results for mitotic chromosomes, we have found no remarkable differences in the C-banding response of these species when examining interphase nuclei of their Malpighian tubules (unpublished data) (Fig. 1).

The Feulgen reaction is a classic cytochemical method useful to distinguish the kinetics of DNA depurination in DNA-protein complexes differing in composition and

organization in the cell nuclei as assessed microspectrophotometrically or by video image analysis (AGRELL and BERGQVIST 1967; MELLO and VIDAL 1980; MELLO 1983; MIYAMOTO *et al*. 2005). Since analysing the somatic interphase nuclei of *M. quadrifasciata* and *M. rufiventris* with the C-banding technique rendered no difference, it was hypothesized that the Feulgen reaction would be better to distinguish the patterns of composition and/or organization of the DNA-protein complexes in interphase cells of these species presumed to differ in terms of heterochromatin content. Feulgen-DNA values obtained at acid hydrolysis times which could distinguish patterns of DNA depurination were thus studied in Malpighian tubule cell nuclei of these *Melipona* species at the same larval phase.

MATERIALS AND METHODS

Fifth instar predefecating larvae from *M. quadrifasciata* Lep. and *M. rufiventris* Lep. (Hymenoptera, Apoidea) reared and supplied by the Federal University of Viçosa (Brazil) were used. Whole mounts of Malpighian tubules obtained from larvae in Ringer solution were fixed in absolute ethanol-glacial acetic acid (3:1, v/v) for 1 min, followed by rinsing in 70% ethanol for 1-5 min. Brain ganglia squashed in a drop of the same fixative were used as source of mitotic chromosomes and as controls for the Feulgen-DNA content in diploid cells. Part of the materials was also subjected to the C-banding technique as a reference for comparison to the results obtained with the Feulgen reaction.

C–banding - Heterochromatin was visualized using the barium/saline/Giemsa (BSG) method (SUMNER 1972), with adaptations for bees. One day after preparation, the slides were immersed in 0.2 N HCl for 6 min and then rapidly rinsed in distilled water

before incubation with 5% $Ba(OH)_2$ at 60°C in a waterbath for 15 min. After rinsing with 0.2 N HCl for approximately 30 s and then in distilled water, the slides were treated with a 2 x SSC solution at pH 7.0 for 15 min at 60°C. The preparations were subsequently stained with Giemsa and treated with 8% Sörensen buffer at pH 6.8 before being air dried. After clearing in xylene, the preparations were mounted in natural Canada balsam and then examined and photographed with a Zeiss Axiophot II microscope (Oberkochen, Germany).

Feulgen staining - The Malpighian tubules were subjected to the Feulgen reaction after treatment with 5% sodium borohydride and acetone–chloroform (1:1, v/v) for 15 min each prior to HCl hydrolysis, in order to prevent a cytoplasmic plasmal reaction (MAMPUMBU *et al*. 2004). Acid hydrolysis was done in 4 M HCl at 25°C for 50 or 90 min followed by treatment with Schiff reagent for 40 min. The hydrolysis times were chosen after a preliminary test which revealed that at the hydrolysis time of 90 min a maximal depurination occurred for the DNA of both species. The preparations were rinsed three times in sulfurous water and once in distilled water before being air dried. The material was then cleared in xylene and mounted in natural Canada balsam (n_D = 1.54). Squashes of brain ganglia subjected to the Feulgen reaction after hydrolysis for 90 min were used as controls of the low ploidy degrees.

Scanning microspectrophotometry image analysis - Images were obtained with a Zeiss automatic scanning microspectrophotometer interfaced to a personal computer. The operating conditions were: Planapo objective 40/0.95; optovar 1.6; measuring diaphragm diameter, 0.16 mm; field diaphragm diameter, 0.20 mm; λ = 565 nm; photomultiplier R-928; scanning spot of 2 µm X 2 µm; and LD–Epiplan 16/0.30 condenser. These parameters

allowed us to determine the Feulgen-DNA values for entire nuclei (A_T) in arbitrary units (A.U.) and also to calculate AAR (= average absorption ratio, a dimensionless parameter that expresses how many times the average absorbance of the condensed chromatin exceeds that of the entire nucleus). AAR = $(Ac/Sc)/(A_T/S_T)$, where Ac is the Feulgen–DNA value for condensed chromatin, Sc is the area covered by condensed chromatin (in μm^2), and S_T is the stained nuclear area (in μm^2) (VIDAL 1984; VIDAL *et al*. 1998). For condensed chromatin definition, the absorbance 0.200 was selected as the threshold cut off point. Measurements were obtained for three specimens of each species.

Statistics - Calculations were done using the Minitab 10® program (Minitab Inc., State College, PA, USA).

RESULTS

Melipona rufiventris showed mitotic chromosomes with strong C-banding that extended along most of their length, except for their ends. In contrast, the chromosomes of *M. quadrifasciata* generally showed weak C-banding, except for a few isolated points (Fig. 1). No difference in chromatin condensation estimated visually by the C-banding technique was found in the interphase nuclei of the Malpighian tubules of the same species or between the compared species (Fig. 1).

The Feulgen-DNA values of the lower class interval (2 C) for the brain ganglia cells of *M. quadrifasciata* (0.24-0.48 A.U.) were larger than those for *M. rufiventris* (0.16-0.32 A.U.) (Fig. 2), and permitted to show that there was more than one ploidy class in the Malpighian tubule cells in each species (Fig. 3), irrespective of the hydrolysis time

considered (*M. rufiventris*, 128 and 256 C classes; *M. quadrifasciata*, 64, 128 and 256 C classes) (Table 1). In addition, after maximal DNA depurination in Malpighian tubule cell nuclei, the Feulgen-DNA values corresponding to 128 and 256 C classes were higher in *M. quadrifasciata* (Fig. 3, Table 1). A similar relationship was observed when the Feulgen-DNA values were estimated after a shorter hydrolysis time (Table 1). However, the Feulgen-DNA values for the majority of the cell nuclear populations of *M. quadrifasciata* did not significantly differ (64 C class) after hydrolysis for 50 and 90 min or, under the hydrolysis time of 50 min, they were 92% from values at the 90 min time (128 C class). As regards the values for *M. rufiventris* after hydrolysis for 50 min, they were always much lower than those obtained after 90 min (128 and 256 C classes); under the hydrolysis time of 50 min Feulgen-DNA values contained in the 128 class were 47% from values at the 90 min time (Table 1).

When the AAR values were compared, nuclei with more contrast between condensed and non-condensed chromatin (AAR > 1.500) were more frequent in *M. rufiventris* (Table 2).

DISCUSSION

The different C-banding patterns seen in the mitotic chromosomes of *M. rufiventris* and *M. quadrifasciata* agreed with findings by ROCHA and POMPOLO (1998) and POMPOLO (2002 – unpublished). However, the C-banding technique was not capable to discriminate chromatin areas with different patterns of condensation in the interphase polyploid nuclei of the Malpighian tubules on the same species considered or when comparing *M. rufiventris* to *M. quadrifasciata.* Probably, the C-banding method was not sensitive enough for this

discrimination in the chromatin of interphase nuclei of the analyzed materials due to a relatively unpacked state of the chromatin in comparison to mitotic chromosomes, and also due to differences in the nucleoprotein complexes involved. Several authors have reported some unexpected results with the classic C-banding techniques in chromosomes; the mechanism of the banding process and the nature of the bands is still a matter for discussion (SUMNER 1990; WALLER and ANGUS 2005).

The simultaneous presence of different degrees of ploidy in the Malpighian tubule cells of fully grown meliponid larvae agreed with a previous report for *M. quadrifasciata* (Mello and Takahashi 1969). The finding that the Feulgen-DNA values for each of the degrees of ploidy in the ganglia and Malpighian tubules of *M. quadrifasciata* were higher than those in *M. rufiventris* indicated differences in DNA amounts in these species, although both have the same number of chromosomes (KERR 1972; TAMBASCO *et al.* 1979; ROCHA and POMPOLO 1998; ROCHA *et al.* 2002).

The finding that the Feulgen-DNA values after the acid hydrolysis for 50 and 90 min are generally much closer in *M. quadrifasciata* than in *M. rufiventris* indicated that DNA depurination occurs faster in *M. quadrifasciata* (MELLO 1983; MIYAMOTO et al. 2005). The faster depurination response in *M. quadrifasciata* may reflect differences in the degree of chromatin packing related to variations in the amount of heterochromatin. On the one hand, this supports the idea that *M. quadrifasciata* interphase nuclei are more easily available to DNA depurination due to its relatively less packed chromatin states (ROCHA and POMPOLO 1998; ROCHA *et al*. 2002). On the other hand, the idea that *M. rufiventris* Malpighian tubule cell nuclei contain more heterochromatin than *M. quadrifasciata* (POMPOLO 2002 – unpublished), although not detectable with the C-banding, is reinforced

by a clear depurination maximum observed at the longer hydrolysis time, as compared to *M. quadrifasciata* results. Additionally, AAR values, which allow the identification of nuclei with different contrast between high and low stainings (VIDAL 1984; VIDAL *et al*.1998), also support this idea. Higher AAR values in *M. rufiventris* interphase nuclei in comparison to those of *M. quadrifasciata* subjected to the Feulgen reaction and studied microspectrophotometrically, are in agreement with a higher heterogeneity in the chromatin packing states of the former, although not detectable visually even with the Feulgen reaction.

ACKNOWLEDGMENTS

This study was suported by the Brazilian Research and Development Council (CNPq) and the State of São Paulo Research Foundation (Fapesp). The authors are indebted to Dr. SÍLVIA G. POMPOLO (Federal University of Viçosa, Brazil) for providing insects and unpublished data, and for reading the manuscript.

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LEGENDS OF FIGURES

Fig 1 - C-banding response in mitotic chromosomes (a, b) and Malpighian tubule cell nuclei (c-f) of *M. quadrifasciata* (a, c, e) and *M. rufiventris* (b, d, f). Since whole-mounted Malpighian tubules were used, nuclei do not appear focused equally. Bars equal 10 μm (a, b, e, f) and 20 μm (c, d).

Fig. 2 - Feulgen-DNA amounts in arbitrary units (A.U.) vs. nuclear area (μm^2) for nuclei of brain ganglia cells from *M. rufiventris* (a) and *M. quadrifasciata* (b) (Hydrolysis time = 90 min; number of specimens = 3).

Fig. 3 - Feulgen-DNA amounts in arbitrary units (A.U.) vs nuclear area (μm^2) regression plots for nuclei of Malpighian tubule cells from *M. rufiventris* (a) and *M. quadrifasciata* (b). (Hydrolysis time = 90 min; number of specimens = 3).

Table 1 - Feulgen-DNA values in arbitrary units (A.U.) for nuclei of each ploidy class in Malpighian tubule cells of *M. rufiventris* and *M. quadrifasciata.*

Species	Hydrolysis times (min)	C classes	Feulgen-DNA values (A.U.) $\bar{X}$	S	n
M. rufiventris	50	128	7.053**A**	1.377	172
		256	13.525**B**	3.806	128
	90	128	15.122**C**	1.713	184
		256	30.261**D**	2.674	116
M. quadrifasciata	50	64	11.353**a**	2.950	223
		128	21.264**b**	2.292	69
		256	37.406**c**	8.222	8
	90	64	11.944**a**	1.528	40
		128	23.124**d**	2.890	231
		256	46.086**e**	7.142	29

C, ploidy degree; n, number of measurements; S, standard deviation; $\bar{X}$, arithmetic mean; 2 C, 0.24-0.48 A.U. – *M. quadrifasciata*; 0.16-0.32 A.U. – *M. rufiventris* (see Fig. 2); different letters down the queue indicate Anova differences significant at the P0.05 level.

Table 2 - Relative frequencies of nuclei in different average absorption ratios (AAR) intervals

	Relative frequency (%)	
AAR interval	*M. rufiventris*	*M. quadrifasciata*
1.0 - 1.5	48.0	60.6
1.501 – 2.5	49.7	35.2
2.501 – 4.0	2.3	3.9
4.001 - 5.0	0.0	0.3

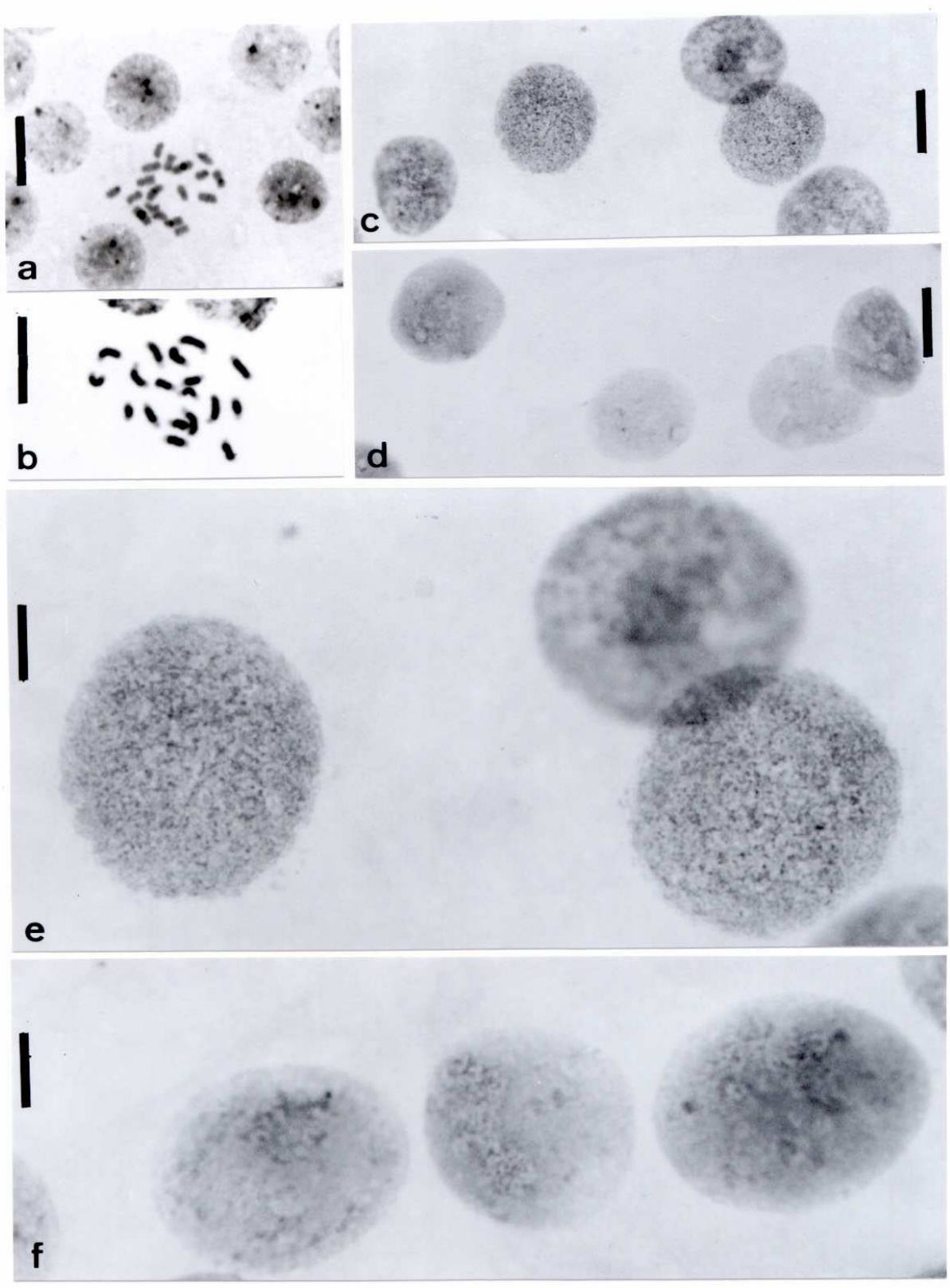
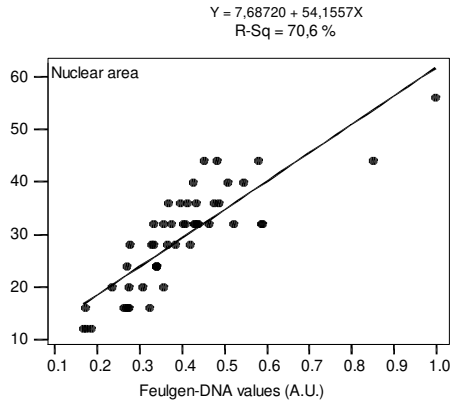


Fig. 1

a


Y = 7,68720 + 54,1557X
R-Sq = 70,6 %
Nuclear area
60
50
40
30
20
10
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Feulgen-DNA values (A.U.)

b

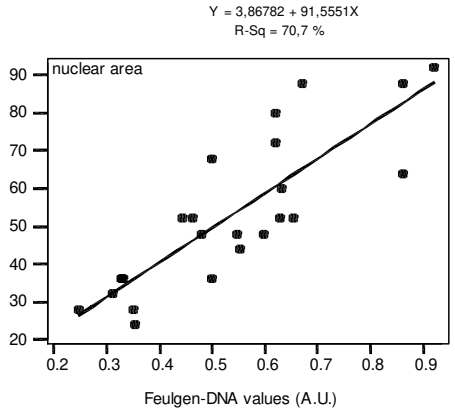
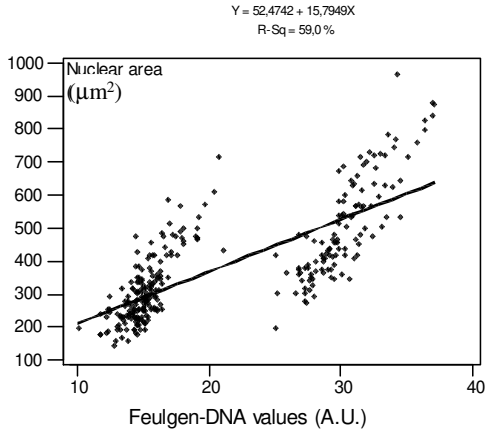

Y = 3,86782 + 91,5551X
R-Sq = 70,7 %
nuclear area
90
80
70
60
50
40
30
20
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Feulgen-DNA values (A.U.)

Fig. 2

a


Y = 52,4742 + 15,7949X
R-Sq = 59,0 %
Nuclear area
(µm²)
1000
900
800
700
600
500
400
300
200
100
10
20
30
40
Feulgen-DNA values (A.U.)

b

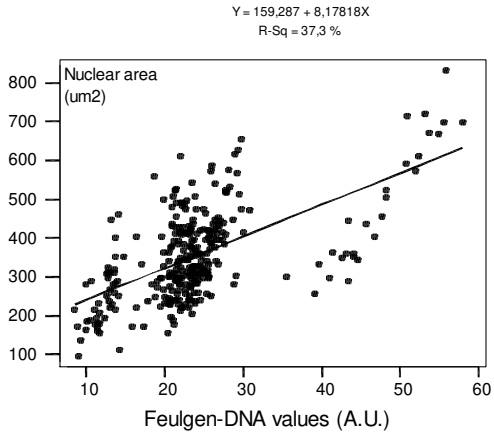

Y = 159,287 + 8,17818X
R-Sq = 37,3 %
Nuclear area
(um2)
800
700
600
500
400
300
200
100
10
20
30
40
50
60
Feulgen-DNA values (A.U.)

Fig. 3

3º Artigo

3. Mampumbu AR. & Mello MLS. DNA methylation in bees with low and high heterochromatin contents as assessed by restriction enzyme digestion and image analysis. Cytometry.

q 2006 International Society for Analytical Cytology Cytometry Part A 69A:000–000 (2006)

DNA Methylation in Stingless Bees with Low and High Heterochromatin Contents as Assessed by Restriction Enzyme Digestion and Image Analysis

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Background: The stingless bee genusMeliponahas been divided into two groups, based on their heterochromatin content.Melipona quadrifasciata and Melipona rufiventris have low and high levels of heterochromatin, respectively. Since condensed chromatin may be rich in methylated DNA sequences,M. quadrifasciataandM. rufiventris nuclei may contain different amounts of methylated CpG. These differences could be assessed by comparing Feulgen-DNA values obtained by image analysis of cells treated with the restriction enzymes Msp I and Hpa II that distinguish between methylated and unmethylated DNA. Msp I and Hpa II cleave the sequence -CCGG-, but there is no cleavage by Hpa II if the cytosine of the central CG dinucleotide is methylated.

Methods: Malpighian tubules of M. quadrifasciata and M. rufiventris were treated with Msp I and Hpa II prior to the Feulgen reaction, and analyzed by automatic scanning microspectrophotometry.

Results: The Feulgen-DNA values for the heterochroma- tin of M. rufiventris and for the small heterochromatin and some euchromatin domains ofM. quadrifasciata mostly decreased after treatment with Msp I, but were unchanged after treatment with Hpa II.

Conclusion: CpG methylation, although detected in diverse chromatin compartments in different bee species, may induce silencing effects required for the same cell physiology. q 2006 International Society for Analytical Cytology

Key terms: DNA methylation; heterochromatin; Malpigh- ian tubules; stingless bees; restriction enzymes; image analysis

The stingless bee genus Melipona has been divided into two groups based on their relative content of heterochromatin revealed by C-banding of mitotic chromosomes (1,2). In chromosomes of Melipona species with a high amount of heterochromatin, euchromatin is restricted to the distal end of the chromosomes (2). High heterochromatin contents, which characterize the group II species of Melipona, are assumed to be evolved via amplification of heterochromatic segments, or by heterochromatin addition (2).

Melipona quadrifasciata (Group I) and Melipona rufiventris (Group II) have the same number of chromosomes but low and high amounts of heterochromatin, respectively (1–3; Pompolo SG, personal communication). However, when the C-banding technique is applied to interphase chromatin such as that present in larval Malpighian tubules of these species, no differences have been found (3). On the other hand, the interphase chromatin of Malpighian tubule cell nuclei of M. quadrifasciata could be distinguished from that of M. rufiventris after their Feulgen reaction kinetics was compared (3). There was indication that the DNA depurination in M. quadrifasciata occurred faster than that in M. rufiventris (3), agreeing with the idea that in interphase nuclei of M. rufiventris the amount of condensed chromatin (heterochromatin) was also more representative than in M. quadrifasciata, although not revealed by the C-banding technique (1–3; Pompolo SG, personal communication).

CG-rich DNA has been found in chromosomes of Melipona species with low and high contents of heterochromatin (2). When CG-rich DNA is present in the heterochromatin of members of Group I Melipona, it appears restricted to the NOR region (2,4).

One of the most common epigenetic modifications in heterochromatin is DNA methylation, which occurs in diverse phylogenetic groups (5), affects chromatin structure, stability, and expression (6–9), and has an important role in many biological processes, including development (5,10,11), genomic imprinting (12), and tissue-specific

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gene expression (13). Since the interphase nuclei of M. rufiventris and M. quadrifasciata differ in their degree of chromatin condensation (3), maybe they also show different levels of DNA methylation.

The occurrence of methylation in DNA -CCGG- sequences in chromosomes and in interphase condensed chromatin can be evaluated in situ after treating chromosomal and chromatin preparations with the isoschizo- meric restriction enzymes Msp I and Hpa II (14–19). Both Msp I and Hpa II cleave the sequence -CCGG-, but Hpa II does not cleave it if the central C of the internal CG dinucleotide is methylated (20). Additionally, image analysis studies have shown that restriction enzymes recognize, cleave, and remove DNA from fixed chromosomes and chromatin, to produce enzyme-specific images (18,19). Image analysis of Feulgen-stained cell preparations treated with Msp I and Hpa II has proven useful to show that the extent of DNA CpG methylation does not influence the higher order remodeling of the chromatin seen following the transformation of NIH 3T3 cells with the Ha-ras oncogene (19).

In this study, the presence of methylated CpG sequences associated with chromatin condensation was investigated by image analysis in Feulgen-stained interphase nuclei of M. rufiventris and M. quadrifasciata, previously treated with Msp I and Hpa II restriction enzymes.

MATERIALS AND METHODS

Fifth instar predefecating larvae of M. quadrifasciata Lep. andM. rufiventrisLep. (Hymenoptera, Apoidea) reared and supplied by the Federal University of Vic¸osa (Vic¸osa, MG, Brazil) were used. Whole mounts of larval Malpighian tubules were fixed in absolute ethanol–glacial acetic acid (3:1, v/v) for 1 min, followed by rinsing in 70% ethanol for 1–5 min.

Digestion with Restriction Enzymes

The Malpighian tubules were initially treated with Tri- ton X-100 (1%) for 2 min to make the nuclear envelope more permeable to enzyme digestion (21,22). Subsequently, the preparations were incubated with 1.0 U/Ilof the restriction enzymes Msp Ior Hpa II (Amersham Biosciences AB, Uppsala, Sweden and Roche Corporation, IN) in appropriate buffers and then covered with coverslips (16,23). The incubations were done in a moist cham- ber at 37LC for 10 h, after which the slides were washed in distilled water and air dried (23). As controls, untreated preparations and tubules treated with Triton X-100 plus the assay buffer for each of the enzymes, in the absence of these enzymes were used . All of the preparations were then processed for the Feulgen reaction.

AQ1

Staining Procedure

The Feulgen reaction involved hydrolysis in 4 M HCl at 25LC for 90 min followed by incubation with Schiff re- agent for 40 min. The acid hydrolysis time corresponded to the maximal depurination as previously reported (3). The Feulgen reaction was preceded by treatment with sodium borohydride followed by acetone–chloroform for 15 min to prevent the cytoplasmic plasmal reaction (24). The material was then cleared in xylene and mounted in Canada balsam (n_D 5 1.54).

Scanning Microspectrophotometry Image Analysis

Images were obtained with a Zeiss automatic scanning microspectrophotometer interfaced to a personal com- puter. The operating conditions were: Planapo objective 40/0.95; optovar 1.6; measuring diaphragm diameter, 0.16 mm; field diaphragm diameter, 0.20 mm; an LD-Epiplan 16/0.30 condenser; scanning spot of 2 Im 3 2 Im; halogen 100-W/12-V lamp, stabilized electronic power supply, Zeiss light modulator, a k of 565 nm obtained with a Schott monochromator filter ruler, an R-928 photomultiplier, and a Pentium II microcomputer. Grid points (individual measuring points) showing absorbances no higher than 0.020 were considered to be background and were automatically removed from the nuclear image. The cutoff point of 0.200 was selected to evaluate the areas covered by condensed chromatin after a preliminary test done using the untreated control. The nuclei were measured individually. The image analysis parameters pertinent to this investigation were:

1. A_T, total integrated absorbance (5 nuclear Feulgen-DNA values in arbitrary units).
2. A_C, integrated absorbance over the preselected cut- off. The same cutoff was maintained for nuclei under the various experimental conditions. A_C corresponds to the "condensed" chromatin Feulgen-DNA values.
3. A_C%, "condensed" chromatin Feulgen-DNA values relative to the nuclear (whole chromatin) Feulgen-DNA values.
4. S_T, nuclear absorbing area in Im^2.
5. S_C, area inIm^2covered with stained chromatin showing absorbances above the selected cutoff point.
6. S_C%, area covered with "condensed" chromatin relative to the nuclear area.
7. AAR 5 $(A_C/S_C)/A_T/S_T)$ 5 average absorption ratio, a dimensionless parameter that expresses how many times the average absorbance of the "condensed" chromatin exceeds that of the entire nucleus (25).

A_C%, S_C %, and AAR were especially used for comparisons, since they were not affected by nuclear ploidy degrees, which vary in the Malpighian tubules of the fifth instar larvae of M. quadrifasciata and M. rufiventris (3).

Scatter diagrams relating AAR to S_C% as previously proposed (26,27) were also plotted. These diagrams allow the discrimination of the position of points corresponding to specific nuclear phenotypes (26–29).

Nuclei from at least three larvae were used for each experimental condition; they were always chosen at random in the Malpighian tubule preparations. The number of nuclei measured under the various experimental conditions was as follows: 300, for untreated controls of M. quadrifasciata and M. rufiventris; 150, for Triton X-100

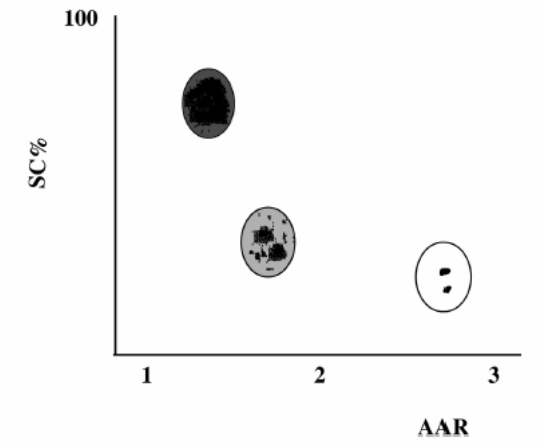

FIG. 1. Scatter diagram representation relating S_C% (relative "con- densed" chromatin area) and AAR (average absorption ration, that is, con- trast between "condensed" and nonabsorbing material) for three different points (Feulgen-stained nuclei); their approximate position is a function of their specific phenotypic images (modified from Vidal et al. (29).

AQ2

plus the Msp I assay buffer condition of M. quadrifasciata; 200, for Triton X-100 plus the Msp I enzyme condition of M. quadrifasciata; and 100, for each of the other conditions used.

Statistics

All calculations were done using the Minitab 10 statis- cal software for Windows (Minitab, State College, PA).

RESULTS

The S_C% versus AAR scatter diagram representation when applied to Feulgen-stained nuclei shows a distribution of points corresponding to nuclei with specific phenotypes defined in terms of their percentages of "condensed" chromatin (S_C%) and contrast between "con- densed" and noncondensed chromatin (AAR) (Fig. 1). In the present study, the analysis of the S_C% versus AAR scatter diagram for the Feulgen-stained control interphase nuclei of M. rufiventris and M. quadrifasciata revealed that the nuclear phenotypes of the Malpighian tubules for both species is practically the same, considering that their "condensed" chromatin areas were defined with a threshold level chosen as 0.200 (Fig. 2). This cutoff point was also certainly responsible for the observation of relative Feulgen-DNA values for the "condensed" chromatin (A_C%) inM. quadrifasciatabecoming unexpectedly elevated (Table 1).

F1

F2

T1

The "condensed" chromatin areas relative to the nuclear area (S_C%) in M. rufiventris, but not in M. quadrifasciata, became smaller after treatment with Triton X-100 followed by Msp Ior Hpa II buffer (enzyme controls), in comparison to untreated controls (Figs. 3 and 4). A certain unpackaging of "condensed" chromatin possibly occurred for M. rufiventris in response to this treatment, since the absolute area covered with "condensed" chromatin (S_C in Im^2) decreased after it (Table 2).

F3,F4

T2

When the scatter diagrams were compared after digestion of the chromatin with the enzymes Msp I and Hpa II, differences with the enzyme type and the species considered were detected (Figs. 3 and 4). Msp I clearly diminished the percentage of "condensed" chromatin area for most cell nuclei in M. quadrifasciata, increasing for these the contrast between the "condensed" and noncondensed chromatin, which remained uncut after removal of the CG-rich DNA (Fig. 3A, Table 3). This indicates a significant presence of CG-rich DNA, capable to be removed with Msp I, in the "condensed" and noncondensed chromatin of the Malpighian tubule cells of M. quadrifasciata. In addition, Hpa II diminished the S_C% values only for part of the nuclear population of the Malpighian tubule cells ofM. quadrifasciata, decreasing the contrast between "condensed" and noncondensed chromatin for them (Fig. 3B, Table 3). Since the position of most of the cell nuclei in M. quadrifasciata with the highest percentages (>50%) of areas covered with a more homogeneously distributed "condensed" chromatin was not changed in the scatter diagram after treatment with Hpa II, the

T3

COLOR

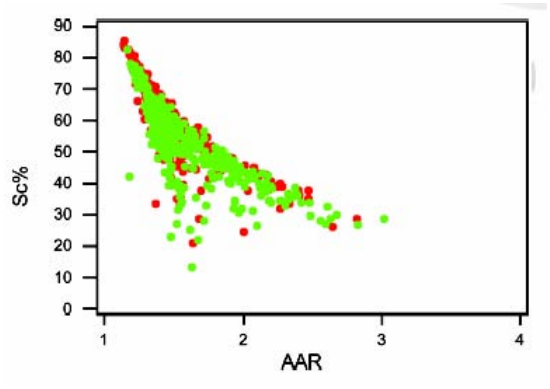

FIG.2. S_C% (relative "condensed" chromatin area) versus AAR (average absorption ratio) scatter diagrams for Feulgen-stained cells of M. quadri- fasciata (red points) and M. rufiventris (green points). No difference in the distribution of points is observed when comparing the two bee species. See Figure 1 for a better understanding of the point positions as associated to specific nuclear phenotypic images.

Table 1
Comparison of Relative Feulgen-DNA Values for "Condensed" Chromatin (A_C%) of Malpighian Tubule Cell Nuclei of 5th Instar Larvae of M. quadrifasciata *and* M. rufiventris

	A_C%			A_C% comparison (Mann-Whitney)	
Species	$\bar{X}$	S	Median	P	Decision
M. quadrifasciata[a]	86.31	9.55	88.85		
M. rufiventris[a]	80.97	11.38	83.67		
M. quadrifasciata vs. *M. Rufiventris*				0.000	SS

[a] $n = 300$; SS, highly significant ($P_{0.01}$).

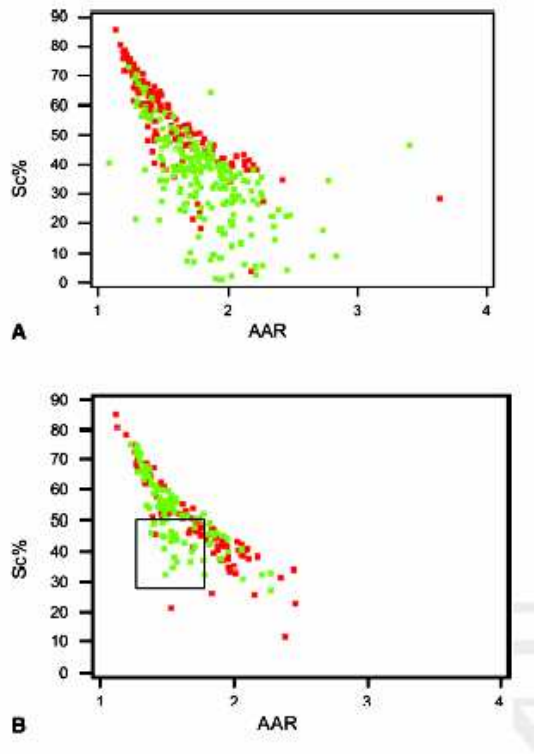

FIG.3. S_C% (relative ''condensed'' chromatin area) versus AAR (average absorption ratio) scatter diagrams for Feulgen-stained cells of M. quadri- fasciata treated with Msp I (green points-A)or Hpa II (green points-B) compared to their respective controls (Triton X-100 plus Msp I (A) or Hpa II (B) assay buffer (red points)). In (A), the general diminishment of S_C% values by Msp I digestion (green points) indicates CG richness in the ''condensed'' chromatin; since there are nuclei with a shift to higher AAR values after this digestion, CG richness should also occur in noncondensed chromatin. In (B), the frame highlights nuclei, which contained CpG sequences not methylated in the internal cytosine of the ''con- densed'' chromatin, since in this case their S_C% and AAR values appear diminished by Hpa II digestion (green points). See Figure 1 for a better understanding of the point positions as associated to specific nuclear phenotypic images.

''condensed'' chromatin of these nuclei probably contain CpG sequences methylated in their internal cytosine.

As regards M. rufiventris, Msp I diminished the ''con- densed'' chromatin percentage area (S_C%) for a significant part of the cell nuclei, slightly increasing for these the contrast between the ''condensed'' and noncondensed chro- matin, which remained uncut after removal of the CG-rich DNA sequences (Fig. 4A, Table 3). This indicates that there are nuclei in the Malpighian tubules of M. rufiventris containing CG-rich DNA in areas of ''condensed'' and noncondensed chromatin, capable to be cut by Msp I. In addition, only the cell nuclei characterized by presenting high S_C% values (>50%) had these values not diminished nor the contrast between ''condensed'' and noncondensed chromatin (AAR) changed, with the Hpa II treatment (Fig. 4B, Table 3); the ''condensed'' chromatin of these nuclei probably contain CpG sequences methylated in their internal cytosine.

DISCUSSION

The results reported here were especially based on the analysis of the distribution of the Feulgen-stained cell nuclei in a scatter diagram that plots relative ''condensed'' chromatin areas (S_C%) versus textural contrast between ''condensed'' and noncondensed chromatin (26–29). This representation is useful, since it is not affected by the degree of nuclear ploidy, which varies, in the Malpighian tubules of M. quadrifasciata and M. rufiventris (3).

Despite the fact thatM. quadrifasciatacontains a slightly higher DNA content than M. rufiventris (3), the untreated controls of both species showed a similar pat-

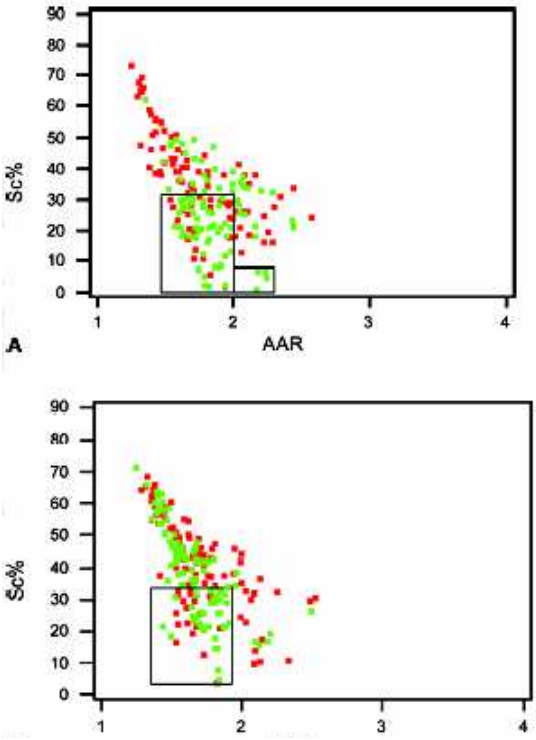

FIG.4.S_C% (relative ''condensed'' chromatin area) versus AAR (average absorption ratio) scatter diagrams for Feulgen-stained cells of M. rufiventris treated with Msp I (green points-A)or Hpa II (green points-B) compared to their respective controls (Triton X-100 plus Msp I (A) or Hpa II (B) assay buffer (red points)). In (A), the frames highlight most nuclei with S_C% values decreased and AAR values slightly increased after Msp I digestion, indicating CG richness in their ''condensed'' chromatin but also modestly in their noncondensed chromatin. In (B), the frame highlights the nuclei with S_C% values diminished and AAR slightly increased by Hpa II digestion (green points); only these nuclei are suggested to contain non-methylated (internal cytosine) CpG sequences in their ''condensed'' chromatin. See Figure 1 for a better understanding of the point positions as associated to specific nuclear phenotypic images.

Table 2
Comparison of Feulgen Absorbing "Condensed" Chromatin Areas (S_C) of Malpighian Tubule Cell Nuclei of 5th Instar Larvae of M. quadrifasciata *and* M. rufiventris *among the Experimental Conditions Analyzed*

Species	Treatments compared	Test	*H*	df	*P*	Decision
M. quadrifasciata	C1, C1-Msp I, C1-Hpa II	Kruskal–Wallis	0.54	2	0.765	NS
M. rufiventris	C2, C2-Msp I	Mann–Whitney	–	–	0.000	SS
	C2, C2-Hpa II	Mann–Whitney	–	–	0.000	SS

C1 and C2, untreated controls; C1-Msp I and C2-Msp I, Triton X-100 + Msp I assay buffer; C1-Hpa II and C2-Hpa II, Triton X-100 + Hpa II assay buffer; df, degree of freedom; NS, nonsignificant; SS, highly significant ($P_{0.01}$).

tern of nucleus distribution (nuclear phenotypes) in the S_C% 3 AAR scatter diagram. This is in agreement with the visual observation of images obtained with the Feulgen reaction (data not shown) or C-banding technique (3). However, since the Feulgen-DNA depurination in interphase cell nuclei of the Malpighian tubules of M. quadrifasciata is faster than that of M. rufiventris, there is indication that M. rufiventris has a higher amount of "condensed" chromatin, at least at the level of its lower-order organization, in comparison toM. quadrifasciata(3), which agrees with the considerations reported for mitotic chromosomes of these species, although based on C-banding results (1–3; Pompolo SG, personal communication).

Present results obtained after using restriction enzymes and microspectrophotometric image analysis indicate occurrence of CpG sequences, capable to be cut with MspI, in the DNA of both "condensed" and noncon- densed chromatin of interphase nuclei of M. quadrifasciata and M. rufiventris, agreeing with the C-banding data for chromosomes ofMeliponaspecies containing high and low amounts of heterochromatin (2). The CG-rich DNA cut by Msp I in the "condensed" chromatin of M. quadrifasciata is certainly contributed by the heterochromatin described to be restricted to the NOR region (2,4), but may also contain euchromatin defined as "condensed" in terms of presenting absorbances higher than 0.200.

Methylated CpG sequences, which remain uncut after Hpa II treatment, were demonstrated in the "condensed" chromatin not only of M. rufiventris, but also of M. quadrifasciata, provided cell nuclei with higher S_C% values are considered. The methylated CpG sequences detected in the Malpighian tubule cell nuclei of M. rufiventris, a spe- cies of Melipona Group II (3; Pompolo SG, personal communication), are assumed to be present predominantly in the heterochromatin, reported to be much more abundant in this species in comparison to M. quadrifasciata (1–3; Pompolo SG, personal communication). Possibly, in the case ofM. quadrifasciata, as mentioned above, "condensed" chromatin means not only heterochromatin, the amount of which is assumed to be less representative in this species (1–3), but also packed euchromatin regions, which became discriminated when the absorbance of 0.200 was used as the cutoff point during microspectrophotometry. In favor of this hypothesis is the fact that the relative Feulgen-DNA values for the "condensed" chroma- tin (A_C%) of untreated cell nuclei of M. quadrifasciata were unexpectedly high.

DNA methylation in complex genomes has been considered to contribute to stability of silenced chromatin states as those found in the heterochromatin (8,9). The methylation of CpG sequences revealed in the heterochromatin of M. rufiventris thus fits in well with this theory. However, after considering that in M. quadrifasciata, methylation in the internal cytosine of CpG sequences occur not only in the less abundant heterochromatin, but also interspersed in packed euchromatin domains, it is suggested that no matter where in the whole chromatin of these different bees the mentioned epigenetic marks are distributed, the silencing effect evolved in specific DNA sequences allowed their Malpighian tubule cells to well accomplish same basic functions. These involve excretion (30) and the secretion of macromolecular components for elaboration of cocoons (31). Although different chromatin constructions may have evolved in different species of the Meliponagenus, they may apparently achieve similar functions in basic terms.

A slight unraveling of the chromatin of M. rufiventris after treatment with Triton X-100 plus the enzyme assay buf- fers, assumed from decrease in S_C% values may be caused by some specificity in composition and supraorganization of this chromatin. Similar findings have been reported for other cell systems (20,32).

Table 3
Relative Frequencies of Nuclei Showing Specific Values of "Condensed" Chromatin Areas (S_C%) versus Average Absorption Ratio (AAR) Changed or Unchanged after Msp I and Hpa II Treatments

	Nuclear frequencies (%)			
	M. quadrifasciata		*M. rufiventris*	
Treatments	S_C%: 0–38, AAR: 1–1.8	S_C%: >50, AAR: 1–1.5	S_C%: 0–38, AAR: 1–1.8	S_C% >50, AAR: 1–1.5
Triton X-100 + Msp I buffer	13.3	24.7	23.0	13.0
Msp I	80.0	7.5	39.0	1.0
Triton X-100 + Hpa II buffer	16.0	15.0	26.0	13.0
Hpa II	25.0	21.0	37.0	12.0

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6 – CONCLUSÕES

1. Montagens totais de túbulos de Malpighi de larvas de *Melipona quadrifasciata* e *Melipona rufiventris* fixados em etanol absoluto-ácido acético glacial (3:1, v/v) por 1 min mostram reação plasmal quando submetidas à reação de Feulgen, por causa de aldeídos de fosfolipídios citoplasmáticos. Esse material requer pré-tratamentos com boroidreto de sódio a 5% e acetona-clorofórmio (1:1, v/v), ambos por 15 min, para que a reação de Feulgen revele apenas DNA.
2. Os núcleos de túbulos de Malpighi de *Melipona quadrifasciata* e *Melipona rufiventris* no 5^{o} estadio larval são poliplóides; em ambas as espécies ocorrem mais de um grau de ploidia.
3. O conteúdo Feulgen-DNA para os núcleos de túbulo de Malpighi do último estágio larval de *Melipona quadrifasciata* é superior ao de *Melipona rufiventris*, embora o número de cromossomos de ambas seja idêntico.
4. Embora a cromatina de células interfásicas dos túbulos de Malpighi de *Melipona quadrifasciata* e *Melipona rufiventris* não mostre diferente resposta à técnica de banda-C, como acontece em cromossomos mitóticos, a depurinação do DNA em *Melipona quadrifasciata* é mais rápida do que em *Melipona rufiventris*, o que confirmaria a diferença em condensação cromatínica entre elas, assumida para cromossomos mitóticos submetido à reação de Feulgen.
5. Usando-se enzimas de restrição, reação de Feulgen e análise de imagem por microespectrofotometria, pode-se determinar *in situ* a ocorrência de seqüências

CpG, capazes de serem cortadas com *Msp* I, na cromatina condensada e na cromatina não condensada de *M. quadrifasciata* e *M. rufiventris*.

6. Seqüências CpG metiladas em sua citosina interna, não susceptíveis à remoção por *Hpa* II, ocorrem na heterocromatina abundante de *M. rufiventris* e na heterocromatina pouca abundante e em áreas eucromáticas compactadas de *M. quadrifasciata*. Essas alterações epigenéticas, embora contidas em compartimentos cromatínicos diversos, conforme a espécie de *Melipona* considerada, trazem um silenciamento que pode estar contribuindo para a realização nos túbulos de Malpighi de mesmas funções celulares básicas, como as de excreção e secreção.