ANDREIA ARANTES BORGES

AVALIAÇÃO DA EXPRESSÃO GÊNICA DIFERENCIAL E DOS HIDROCARBONETOS CUTICULARES DE RAINHAS, OPERÁRIAS, MACHOS HAPLÓIDES E DIPLÓIDES DE *Melipona quadrifasciata* (HYMENOPTERA: APIDAE)

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento, para obtenção do título de *Doctor Scientiae*.

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Profº Lucio Antonio de Oliveira Campos (Coorientador)	Prof° Klaus Hartfelder (Coorientador)
Profº José Eduardo Serrão	Profª Denilce Meneses Lopes

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(Orientadora)

Há pessoas que nos falam e nem as escutamos;
Há pessoas que nos ferem e nem cicatrizes deixam.
Mas há pessoas que, simplesmente, aparecem em nossa vida
E que marcam para sempre...

Cecília Meireles

À minha família amada,
em especial à minha
She Mary e Papito.

Novamente dedico.

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BIOGRAFIA

Andreia Arantes Borges, filha de Divino Marques Borges e Maria Arantes Borges, nasceu em 19 de abril de 1982, na cidade de Formiga, Minas Gerais.

Concluiu seus estudos de primeiro e segundo graus nesta cidade na Escola Estadual Drº Abílio Machado.

Em 2001, ingressou-se na Universidade Federal de Viçosa (UFV), graduando-se bacharel e licenciada no curso de Ciências Biológicas em julho de 2005.

Foi bolsista de iniciação científica (PIBIC-CNPq) no período de 2002 a 2005, desenvolvendo trabalhos relacionados a biologia molecular de abelhas, no Departamento de Biologia Geral, sob orientação da Profª. Mara Garcia Tavares.

Em agosto de 2005, iniciou o curso de Mestrado em Genética e Melhoramento, na Universidade Federal de Viçosa, submetendo-se à defesa da dissertação em 13 de março de 2007, sob orientação da Profª Mara Garcia Tavares.

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RESUMO

BORGES, Andreia Arantes, D.Sc., Universidade Federal de Viçosa, junho de 2011. **Avaliação da expressão gênica diferencial e dos hidrocarbonetos cuticulares de rainhas, operárias, machos haplóides e diplóides de *Melipona quadrifasciata* (Hymenoptera: Apidae).** Orientadora: Mara Garcia Tavares. Coorientadores: Lucio Antonio de Oliveira Campos e Klaus Hartfelder.

No presente trabalho procurou-se abordar diferentes aspectos relacionados a expressão gênica e composição dos hidrocarbonetos cuticulares de rainhas, operárias, machos haplóides e machos diplóides de *Melipona quadrifasciata,* assim como se procurou compreender os mecanismos moleculares envolvidos nos processos de diferenciação dos machos desta abelha. O perfil de expressão gênica de dez genes casta-específicos foi avaliado de forma comparativa entre operárias, rainhas, machos haplóides e diplóides recém-emergidos e com cinco dias de idade adulta. De modo geral, a abundância de transcritos de machos diplóides foi mais similar à de machos haplóides e operárias do que à de rainhas, para as duas classes etárias analisadas. Após cinco dias de emergência verificaram-se aumentos significativos nos níveis de expressão gênica de rainhas e operárias, enquanto que resultados opostos foram observados nos machos. Estas diferenças podem estar relacionadas à longevidade diferencial de machos e fêmeas. Apesar dos machos haplóides e diplóides não serem facilmente distinguidos visualmente, genes diferencialmente expressos entre estes dois fenótipos foram identificados por meio da técnica de RDA (*Representational Difference Analysis*). Na biblioteca obtida de machos haplóides, observou-se que 32.5% das ESTs apresentaram similaridade às sequências de *Drosophila,* 25.6% às sequências de *Apis* e 7% são similares à meliponíneos. Na biblioteca de machos diplóides, as porcentagens de similaridade às sequências de *Drosophila* e *Apis* foram levemente maiores, sendo 41.8% e 30.2%, respectivamente. Em ambas as bibliotecas subtrativas, algumas ESTs não apresentaram similaridade significativa com nenhuma sequência depositada em bancos de dados públicos sendo consideradas como produtos preditos (34.9% e 28% para as bibliotecas de machos

haplóides e diplóides, respectivamente). A expressão gênica diferencial foi confirmada por PCR em tempo real para dez de dezessete genes selecionados. Análises de expressão gênica comparativa com amostras de machos com cinco dias de idade revelaram mudanças na abundância dos transcritos neste período em que ocorre a maturação sexual e o início da migração dos espermatozóides dos testículos para a vesícula seminal. Machos e fêmeas de *M. quadrifasciata* apresentaram diferentes assinaturas química cuticulares, sendo o padrão químico de machos haplóides e diplóides mais similares aos de operárias do que aos de rainhas, corroborando as semelhanças morfológicas e comportamentais entre machos e operárias. Nenhum composto foi encontrado exclusivamente na cutícula dos machos diplóides recém-emergidos, indicando a inexistência de uma substância que ative o comportamento agressivo das operárias quando estes machos são detectados em colônias endogâmicas.

ABSTRACT

BORGES, Andreia Arantes, D.Sc., Universidade Federal de Viçosa, June of 2011. **Evaluation of differential gene expression and cuticular hydrocarbons of queens, workers, haploid and diploid males of *Melipona quadrifasciata* (Hymenoptera: Apidae).** Adviser: Mara Garcia Tavares. Co-Advisers: Lucio Antonio de Oliveira Campos and Klaus Hartfelder.

The present study had focused to address different aspects of gene expression and cuticular hydrocarbons composition of queens, workers, diploid and haploid males of *Melipona quadrifasciata*, as well as trying to understand the molecular mechanisms involved in the process of differentiation of male. The profile of gene expression of ten caste-specific genes was evaluated comparatively among newly emerged and 5 day-old workers, queens, diploid and haploid males. In general, the abundance of transcripts of diploid males was similar to that of haploid males and workers than to queens for the two age groups analyzed. After five days of emergence there were significant increases in gene expression levels of queens and workers, while opposite results were observed in male. These differences may be related to differential longevity between males and females. Despite haploid and diploid males are not easily distinguished visually, differentially expressed genes between these two phenotypes were identified by the technique of RDA (Representational Difference Analysis). Overall, the ESTs sequenced from the haploid male RDA library showed similarity with sequences of *Drosophila* (32.5%), *Apis* (25.6%) and stingless bees (7%). In the diploid male library, the percentage of ESTs with similarity to *Drosophila* and *Apis* sequences were slightly higher, being 41.8% and 30.2%, respectively. Both subtracted libraries also presented considerable proportions of ESTs without significant match to sequences in the nr database (34.9% for the haploid and 28% for the diploid male libraries). Differential gene expression was confirmed by real time PCR for ten of seventeen selected genes. Comparative gene expression analysis on samples of males with five days of age showed changes in abundance of transcripts in the phase of sexual maturation and early migration of sperm from the testicles to the

seminal vesicle. Males and females of *M. quadrifasciata* had different chemical cuticular signatures, and the chemical standard of haploid and diploid males are more similar to workers than those of queens, corroborating the morphological and behavioral similarities between males and workers. No compound was found exclusively in the cuticle of newly emerged diploid males, indicating the absence of a substance that activates the agressive behavior of workers when these males are detected in inbred colonies.

INTRODUÇÃO GERAL

As abelhas desempenham importante papel nas comunidades biológicas, constituindo o principal grupo de polinizadores das angiospermas em diversos ecossistemas (Bawa, 1990; Nef e Simpson, 1993). Deste modo, estes insetos têm reconhecida ação na perpetuação de espécies silvestres, contribuindo para a manutenção do equilíbrio ecológico (La Salle e Gauld, 1993).

Por meio da polinização, as abelhas contribuem para a manutenção do ciclo de reprodução sexuada de grande número de espécies vegetais e, assim, contribuem também para a disponibilidade de alimento para outros animais que dependem das plantas por elas polinizadas (Campos, 1998).

As abelhas Meliponini, popularmente chamadas de “abelhas indígenas sem ferrão”, compreendem centenas de espécies distribuídas nas regiões tropicais e subtropicais do mundo, sendo mais diversas e abundantes nas regiões tropicais (Roubik, 1989). A grande importância das espécies desta tribo está centrada no gênero *Melipona*, que têm distribuição exclusiva na região neotropical (Michener, 1974; 2000).

Em Minas Gerais, estima-se que a fauna de abelhas silvestres englobe algo em torno de 1000 espécies (Almeida e Silveira, citado por Costa *et al*., 1998). Entre estas, encontra-se a espécie *Melipona quadrifasciata*, popularmente conhecida como mandaçaia, a qual pode ser encontrada ao longo da costa dos estados brasileiros, da Paraíba ao Rio Grande do Sul (Moure e Kerr, 1950).

Nas abelhas, assim como na maioria dos Hymenoptera, ovos fertilizados dão origem a fêmeas, ovos não fertilizados originam machos haplóides e ovos fertilizados que apresentam homozigose no loco da determinação sexual se desenvolvem em machos diplóides (Whiting, 1939).

A base genética da determinação do sexo nos organismos haplodiplóides, entretanto, é pouco compreendida e parece ser diversa. O seu entendimento foi inicialmente elucidado pela descoberta dos machos diplóides na descendência de cruzamentos endogâmicos da vespa *Bracon hebetor*. A presença destes machos foi explicada através da hipótese de determinação complementar do sexo – CSD (*Complementary Sex Determination*) (Whiting,

1943). Segundo esta hipótese, indivíduos heterozigotos no loco da determinação sexual desenvolvem-se em fêmeas, enquanto indivíduos que são hemizigotos (haplóides) são machos. Machos diplóides se desenvolvem a partir de ovos fertilizados que carregam os mesmos alelos no loco de determinação do sexo sendo, portanto, homozigotos nesse loco.

Desde a descoberta de Whiting, o sistema CSD foi encontrado em mais de 60 espécies de Hymenoptera, incluindo espécies economicamente importantes como as abelhas melíferas, várias espécies de *sawflies* e vespas (van Wilgenburg *et al*., 2006). Tem se verificado que o gene *csd* apresenta moderada homologia ao gene *transformer* (*tra*), um gene de expressiva importância na determinação do sexo em *Drosophila melanogaster*. Em *Drosophila* o gene *tra* está envolvido no processo de *splicing* fêmea-específico do transcrito do gene *doublesex* o qual inicia a via de desenvolvimento de fêmeas nesta espécie (Cline e Meyer, 1996). Portanto, acredita-se que o gene *csd* participe de forma semelhante ao gene *tra* na determinação do sexo em *Apis*.

Hasselmann *et al*. (2008) detectaram um novo componente na via de determinação do sexo em *Apis*: o gene feminizer (*fem*), localizado a 12 kb *upstream* de *csd*. A repressão dos transcritos do gene *fem* nas fêmeas resulta em uma mudança de desenvolvimento onde a morfologia da cabeça das fêmeas é alterada, assemelhando-se à morfologia típica de machos. Entretanto, o mesmo tratamento em machos diplóides não afeta o desenvolvimento das suas cabeças. Resultados semelhantes foram obtidos com o silenciamento do gene *csd* em machos e fêmeas. Estes dados indicam que o gene *fem* está relacionado com a via de determinação do sexo.

Este sistema de determinação do sexo sugere que o endocruzamento aumenta a proporção de machos diplóides, já que o número de alelos sexuais em homozigose aumenta com este tipo de acasalamento. Contudo, o sistema CSD não é capaz de explicar a determinação do sexo em todos os himenópteros e, outros modelos foram propostos: modelo de balanço gênico (Cunha e Kerr, 1957; Kerr e Nielsen, 1967); determinação do sexo por fertilização (Whiting, 1960); efeito materno (Crozier, 1977) e *imprinting* genômico (Poiré *et al*., 1993).

Independentemente do modelo utilizado, o sistema de determinação responsável pela produção de machos diplóides é sempre desvantajosa impondo um custo significativo ao sucesso reprodutivo de seus progenitores e ao próprio desenvolvimento da colônia (Page, 1980; Ross *et al*., 1993).

Estes machos são estéreis (Camargo, 1979) e até mesmo inviáveis, dependendo da espécie estudada (Stouthamer *et al.*, 1992; Agoze *et al*., 1994). Em *Apis mellifera,* espécie com alimentação progressiva da cria, esses machos são mortos pelas operárias na fase larval (Woyke, 1963). Nos gêneros *Melipona* e *Bombus*, espécies com aprovisionamento massal, os machos diplóides emergem como adultos, sendo mortos posteriormente pelas operárias. Nessas ocasiões, a rainha responsável pela produção dos machos diplóides pode ser morta pelas operárias (Ratnieks, 1990). Segundo Camargo (1982), machos diplóides de *M. quadrifasciata* têm viabilidade normal durante o estágio de desenvolvimento, mas sua longevidade máxima é de 17 dias, contrastando com a longevidade máxima de machos haplóides (30 dias) e de operárias (90 dias).

As características desvantajosas apresentadas pelos machos diplóides podem estar associadas à diferentes níveis de expressão gênica e não apenas devido à homozigose do loco sexual. Desta forma, a identificação dos genes diferencialmente expressos nestes machos assim como nos machos haplóides, rainhas e operárias é importante para conhecer não somente sua função gênica, mas também para compreender como os mecanismos moleculares estão relacionados aos processos biológicos deste grupo de organismos.

Várias metodologias têm sido desenvolvidas para a identificação de genes expressos diferencialmente entre duas ou mais amostras biológicas distintas, entre as quais se destacam: DD-PCR (*Differential Display PCR*, Liang e Pardee, 1992), SAGE (*Serial Analysis of Gene Expression*, Velculescu *et al*., 1995), Microarranjos (DNA *microarray,* Schena *et al*., 1995), SSH-PCR (*Suppressive Subtractive Hybridisation-Polymerase Chain Reaction*, Diatchenko *et al*., 1996) e RDA (*Representational Difference Analysis*, Hubank e Schatz, 2000).

Estas tecnologias têm sido amplamente empregadas na análise da expressão gênica diferencial em abelhas. Contudo, a metodologia de RDA

apresenta a vantagem de permitir a comparação direta da expressão gênica, focalizando em genes específicos por meio da normalização dos níveis dos respectivos mRNAs. Na RDA, tem-se uma seleção positiva de diferenças expressas, através de um processo que envolve a subtração das representações por hibridação. Durante este processo, o material comum é seletivamente eliminado e um produto diferencial é amplificado.

Judice *et al*. (2006) empregaram esta metodologia para avaliar a expressão gênica diferencial nas castas de rainhas e operárias de *M. quadrifasciata*. A maioria das seqüências anotadas foi similar a genes de função desconhecida em *A. mellifera* e verificou-se ainda uma variação no perfil de expressão do conjunto de genes expressos em rainhas tratadas com um análogo do hormônio juvenil quando comparado a rainhas naturais (Judice *et al*., 2006).

A estratégia da hibridização subtrativa foi utilizada com sucesso em outros insetos sociais, como no cupim *Cryptotermes secundus* para identificar genes, envolvidos no controle e regulação da divisão de trabalho, diferencialmente expressos entre rainhas e operárias (Weil *et al*., 2009; 2010). Oppelt *et al*. (2010) construíram bibliotecas subtrativas de glândulas acessórias de machos da formiga *Leptothorax gredleri* utilizando o protocolo RDA e sugeriram que os genes encontrados representam novos genes específicos desta espécie.

Análises de RDA também foram empregadas para identificar genes diferencialmente expressos nas glândulas de muco de zangões de *A. mellifera* durante o processo de maturação sexual. Uma variedade de ESTs foi obtida e destas, três correspondem a genes codificadores de proteínas preditas com possível função reprodutiva (Colonello-Fattini e Hartfelder, 2009).

Atualmente, tem-se aumentado o interesse na identificação de genes diferencialmente expressos em abelhas. Contudo, a maioria dos estudos é realizado com *Apis mellifera*, que constitui um organismo modelo para os estudos genômicos em abelhas.

Cientes da importância ecológica e econômica das abelhas do gênero *Melipona*, o presente trabalho analisou a expressão gênica diferencial de rainhas, operárias, machos haplóides e diplóides da espécie *M. quadrifasciata*

de forma a ampliar o conhecimento sobre expressão gênica nas abelhas pertencentes a este gênero.

Outra abordagem que merece destaque no estudo destas abelhas é o perfil dos hidrocarbonetos cuticulares. A partir deste tipo de análise é possível verificar similaridades e diferenças na composição química da cutícula dos organismos que compõem a colônia.

Estas substâncias cuticulares têm a importante função de atuarem como barreira de proteção contra a desidratação e regulação da permeabilidade cuticular (Lockey, 1988). Além disso, os hidrocarbonetos cuticulares atuam como feromônios moduladores e desencadeadores de comportamentos e a sua ocorrência mostra-se característica entre grupos de insetos, podendo variar dentro de um grupo específico de acordo com o estágio de desenvolvimento, casta ou sexo dos indivíduos (Blomquist *et al.,* 1998; Monnin e Peeters, 1999; Sledge *et al.,* 2001; Boomsma *et al*., 2003).

Abdalla *et al*. (2003) detectaram diferenças qualitativas e quantitativas na composição cuticular de rainhas, operárias e machos de *M. bicolor*. Operárias e rainhas recém-emergidas apresentaram perfis cuticulares similares entre si, porém diferentes daqueles apresentados pelos machos. Em contraposição, Kerr *et al*. (2004) verificaram que os padrões de hidrocarbonetos cuticulares de operárias de *M. scutellaris* são mais similares aos de machos do que aos de rainhas. Estes resultados concordam com os dados morfológicos de que operárias de meliponíneos são mais semelhantes a machos que a rainhas (Kerr, 1974; Kerr e Cunha, 1990).

Diferenças interespecíficas foram encontradas na composição dos hidrocarbonetos cuticulares, extratos abdominais e cefálicos de *Nannotrigona testaceicornis* e *Plebeia droryana*. Alguns compostos, como por exemplo, o fenil tridecano, foi encontrado apenas na cutícula de machos, permitindo distinguí-los das operárias destas espécies (Pianaro *et al*., 2009).

Comparações do perfil químico cuticular entre operárias de diferentes idades e que desempenham diferentes funções nas colônias de *Frieseomelitta varia*, *Schwarziana quadripunctata* e *Melipona marginata* revelaram a existência de diferenças tarefa-específicas (Nunes *et al*., 2009a e b; Ferreira-Caliman *et al*., 2010).

A análise dos padrões de hidrocarbonetos cuticulares tem um cunho genético, pois as substâncias cuticulares produzidas são um reflexo dos genes ativados para sintetizar as enzimas responsáveis pela produção destes importantes compostos. Desta forma, a caracterização destes compostos cuticulares de rainhas, operárias, machos haplóides e diplóides de *M. quadrifasciata* auxiliará indiretamente na compreensão da expressão gênica diferencial.

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OBJETIVO

Este trabalho teve como objetivo geral identificar diferenças no padrão de expressão gênica e dos hidrocarbonetos cuticulares de rainhas, operárias, machos haplóides e diplóides de *Melipona quadrifasciata*.

CAPÍTULO 1

Transcript levels of ten caste-related genes in adult diploid males of *Melipona quadrifasciata* (Hymenoptera: Apidae) – a comparison with haploid males, queens and workers*

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Running title: Gene expression in diploid males

Key words: stingless bee; real time PCR; caste; diploid male; differential gene expression

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Abstract

In Hymenoptera, homozygosity at the sex locus results in the production of diploid males. In social species, these pose a double burden by having low fitness and drawing resources normally spent for increasing the work force of a colony. Nevertheless, studying diploid males is of interest to elucidate effects of ploidy (normal males are haploid, whereas the female castes, the queens and workers, are diploid) on morphology, and life history. Herein we investigated expression levels of ten caste-related genes in the stingless bee *Melipona quadrifasciata*, comparing newly emerged and 5-day-old diploid males with haploid males, queens and workers. In diploid males, transcript levels for *dunce* and *paramyosin* increased while those for *diacyl glycerol kinase* and the transcriptional co-repressor *groucho* diminished during the first five days of adult life. Two general trends were apparent, (i) gene expression patterns in diploid males were overall more similar to haploid males and workers than to queens, and (ii) in queens and workers, more genes were up-regulated from emergence to day 5, whereas in diploid and especially so in haploid males more genes were down-regulated. This difference between the sexes may be related to longevity, which is much higher in females than in males.

Introduction

Sex and caste determination in stingless bees are long debated issues, ever since a genetic predetermination to the female caste phenotypes, the queens and workers, has been proposed for the genus *Melipona* (Kerr, 1948, 1950), contrasting with the more common mode of caste determination in social Hymenoptera which is based on differential nutrition of the larvae (for review see Hartfelder and Engels, 1998; Hartfelder *et al.*, 2006). Though still controversial, because the genetic loci underlying the caste determination mechanism have not yet been mapped and because in terms of ultimate causes the frequencies of queen/worker ratios observed in colonies can also be explained in terms of an optimization model derived from caste-conflict theory (Ratnieks, 2001; Wenseleers and Ratnieks, 2004), genetic caste determination in the genus *Melipona* is no longer an exception, as similar cases have now also been evidenced in several ant species (Fraser *et al.*, 2000; Julian *et al.*, 2002).

Leaving aside its initial triggers, the differentiation of the caste phenotypes of social Hymenoptera is an integral part of postembryonic development. Best analyzed in the honey bee, *Apis mellifera*, queen/worker differentiation is driven by epigenetic factors (Kucharski *et al.*, 2008) and the endocrine system, especially so juvenile hormone an ecdysteroid titers (Rachinsky *et al.*, 1990) and insulin signaling (Wheeler *et al.*, 2006; Azevedo and Hartfelder, 2008).

While these differentiation processes and roles of the queen and worker castes in division of labor within colonies of highly eusocial bees are fairly well understood, relatively little is known about the third morph present within colonies, the males. As in all Hymenoptera, and first shown around 1840 by the Polish priest and bee researcher Johan Dzierzon, the males of bees develop from unfertilized eggs, in contrast to females which are diploid and developing from fertilized eggs. The mechanism underlying sex determination in haplodiploid Hymenoptera was first described by Whiting (1943) in the parasitic wasp *Bracon hebetor*, as a series of multiple alleles at a single sex locus that functionally interact in complementary sex determination (CSD). Heterozygosity at this locus is the genetic basis for

female development, whereas hemi- or homozygosity leads to males. While haploidy, and consequent hemizygosity at the sex locus is the primary cause for male development, the occurrence of diploid males has been noted in several hymenopteran species (van Wilgenburg *et al.*, 2006) as a result of latent inbreeding and consequent allelic matches at the sex locus. The *csd* locus has been mapped and sequenced in the honey bee (Beye *et al.*, 2003) and was later shown to be highly variable within honey bee populations as a result of positive selection (Hasselmann and Beye, 2004). It represents the input to the early embryonic sex determination pathway that has as its output alternatively spliced male and female Doublesex (Dsx) proteins (Gempe *et al.*, 2009).

Allelic matching at the *csd* locus, occurring either naturally as a consequence of loss of genetic variability within a honey bee population, or as experimentally imposed through brother-sister mating or even more so by instrumental insemination of an unmated queen with sperm from one of her own sons, leads to the development of 50% of the fertilized eggs as diploid males. Due to this significant loss in the worker force, diploid male production represents a burden to colony growth and productivity (Page Jr and Laidlaw, 1982; Woyke, 1984). This long term, primary cost can only be eliminated through queen replacement. In contrast, a secondary cost factor, which is the consumption of valuable larval food by diploid male larvae can be reduced, and honey bees do so quite efficiently by removing diploid males at a very early larval stages. Nursing workers perceive these through a “cannibalization signal” (Woyke, 1967), which is thought to be an altered cuticular hydrocarbon profile of the diploid male larvae (Santomauro *et al.*, 2004).

Such early removal of diploid males is not an option for stingless bees, which mass provision brood cells and seal these as soon as an egg has been laid on top of the larval food in a behavioral sequence known as provisioning oviposition process (POP), which is elementary kernel in stingless bee reproductive biology (Zucchi *et al.*, 1999). While not having the early diploid male removal option is a disadvantageous developmental constraint for stingless bees, it provides an opportunity for studying the biology of diploid males in highly eusocial bees, as it does not require cumbersome *in vitro*

rearing of larvae, as is necessary in honey bees (Woyke, 1963a, b). Diploid males of *Melipona quadrifasciata* were first successfully produced by Camargo (1974, 1979) through brother-sister matings, and this has aided research on their life history (Camargo, 1982).

Behavioral, morphological, and cuticular chemical profile comparisons between haploid males and females of stingless bees have shown that meliponine males are more similar to workers than to queens (Campos *et al.*, 1979; Almeida, 1985; Bonetti and Kerr, 1985; Kerr *et al.*, 2004). Furthermore, males of stingless bees have occasionally been seen to participate in colony maintenance activities, such as food exchange and nectar dehydration (Imperatriz-Fonseca, 1973; van Veen *et al.*, 1997).

Hardly anything is known about the behavior of diploid males of stingless bees, primarily because they cannot be distinguished by eye from the normal haploid ones; this being only possible through *post mortem* karyotyping. But they are reported to have shorter life spans (Camargo, 1982), smaller testes, and less sperm (Tavares *et al.*, 2003). Smaller testes and lower sperm numbers have also been denoted in diploid drones of honey bees (Woyke, 1973; Chaud-Netto and Kerr, 1980), as well as in bumble bees (Duchateau and Mariën, 1995).

The lack of morphological variation between haploid and diploid males on the one hand, and the differences in life span and fertility on the other made us ask whether such discrepancies might be reflected in, and thus assessed through gene expression patterns. In the present study we investigated the expression levels of ten genes previously identified as differentially expressed in the female castes of the stingless bee *Melipona quadrifasciata* (Judice *et al.*, 2004; Judice *et al.*, 2006), comparing transcription in diploid males to that of haploid ones, as well as to queens and workers. Real time RT-PCR assays were run on two time points in the adult life cycle, shortly after the bees emerged from the brood combs and when they were five days old. We chose to look at this early window in adult life because it is the period where adult sexual maturation occurs, including the start of migration of spermatozoids from the testes to the seminal vesicles (Camargo, 1984). Furthermore, it is the window of major changes in the endocrine system of *M. quadrifasciata* males, such as a sharp peak in the

ecdysteroid hemolyph titer, occurring in conjunction with a broader peak in the juvenile hormone titer (Santana and Hartfelder, unpublished results).

Material and methods

Bees and total RNA extraction

Males and females of stingless bee *Melipona quadrifasciata* were collected from colonies at the meliponary of the Federal University of Viçosa, Viçosa, MG, Brazil. Haploid males, workers and queens were retrieved from combs taken from non-inbred colonies, whereas diploid males were obtained through inbreeding, from colonies set up from a brother-sister mating scheme (Camargo, 1974, 1979). The ploidy of these males was determined by cytogenetic analysis according to Imai *et al.* (1988).

Brood combs containing late pupal stages were removed from the colonies and kept in an incubator at 28^{o}C. Newly emerged bees were collected in intervals of 4 to 6 hours as they emerged from the brood cells. In order to assess transcript abundance in 5-day-old adults, newly emerged bees were kept in Petri dishes in an incubator (28 ^{o}C) with *ad libitum* access to sugar syrup (50%) and fermented pollen (Camargo, 1979). All individuals were snap frozen in liquid nitrogen and stored at -80^{o}C.

For RNA extraction, two individuals of each type and age class were pooled and homogenized in 1 mL of TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The resultant RNA pellet was resuspended in diethyl pyrocarbonate-treated water. A DNase treatment (0.1U DNAse I, Invitrogen) was done to eliminate potential genomic DNA contamination.

All RNA samples (five per phenotype and age class) were assayed for purity, RNA integrity and concentration through spectrophotometric determination of OD_{260}/OD_{280} ratios, these ranging from 1.8 to 2.0, and electrophoresis in agarose gels under denaturing conditions. Subsequently, the samples were stored at -80 °C for quantitative real time PCR (RT-qPCR) analysis.

Primer design and RT-qPCR analysis

In the present study, we analyzed the transcript levels of ten genes. Those encoding a transmembrane transporter (TM-transporter), a ceramide kinase, a permease and a gene of unknown function, provisionally named Mq5, were chosen from a gene set revealed as differentially expressed in a custom-made microarray for *M. quadrifasciata* (Judice, Pereira and Harttfelder, unpublished). EST clones for these genes were re-sequenced and gene identification checked by blastx and blastn searches against GenBank sequences and the dbEST database for *M. quadrifasciata*. Subsequently, gene specific primers were designed by means of the Primer 3 and Gene Runner version 3.05 softwares. The other six genes included in the study were those encoding dunce, paramyosin, groucho, an amino acid-polyamine transporter (AAP-transporter), a fatty acid synthase (FAS) and diacylglycerol kinase (DGK), which had already been validated as differentially expressed in newly emerged M. quadrifasciata queens and workers (Judice et al., 2006). All PCR primer sequences are listed in Table 1.

First strand cDNA templates were synthesized from 5 µg of DNAse-treated RNA using Superscript II (Invitrogen) reverse transcriptase and oligo $(dT)_{12\text{-}18}$ primer (Invitrogen). Subsequently, optimal cDNA quantities were established for both target and reference genes (*ribosomal protein 49* and *actin).* Serial dilution series made from PCR products of the ten genes were used to calculate primer efficiencies, defined as efficiency = $10(^{-1/slope})$ (Pfaffl, 2001).

Each reaction mixture contained 7 µL SYBR Green (Applied Biosystems), 1 µL of cDNA (diluted 1:10), 0.8 µL of each gene-specific forward and reverse primer (10 pmol/µL) in a final volume of 14 µL. PCR amplifications were done in an ABI Prism 7500 system (Applied Biosystems) with the following thermal cycling profile: 50 °C for 2 min, 95 °C for 10 min, followed by 40 steps of 95 °C for 15 s and 60 °C for 1 min. After 40 amplification cycles, all samples were submitted to dissociation curve analysis to confirm the absence of nonspecific products and primer dimers. In each run, a non-template reaction was included as negative control. None of the negative control samples showed a fluorescence signal, confirming that

the extraction procedure and DNase treatment were efficient in removing residual genomic DNA. Two inter-run calibrators were included in each plate to correct for run-to-run variation, as suggested in the MIQE recommendations (Bustin *et al.*, 2009). We analyzed a total of 40 RNA samples, 20 for each age class, and these being divided into five from each phenotype (diploid and haploid males, queens and workers). All samples were analyzed in triplicate to assure repeatability.

Fold variation in transcript levels was calculated using the comparative Ct method (Pfaffl, 2001). Data were analyzed using the REST program (Pfaffl *et al.*, 2002), which uses a pairwise fixed reallocation randomization testing procedure on expression ratios among treatments. *P* values ≤ 0.05 were considered as statistically significant.

Results

In a first step, we investigated changes in transcript levels that occurred during the first five days of adult life in diploid males (Figure 1A), haploid males (Figure 1B), workers (Figure 1C) and queens (Figure 1D). The box and whisker plots show the expression ratio (fold change) for each gene at day 5 in relation to day 0, i.e. the time point when the bees were emerging from their brood cells.

In diploid males, two genes, *dunce* and *paramyosin* were significantly up-regulated (REST, P ≤ 0.05), whereas *diacylglycerol kinase (DGK)* and groucho expression was down-regulated. In haploid males, two genes, *fatty acid synthase (FAS)* and again *groucho* were significantly less expressed. In workers, the transcript levels of *transmembrane transporter (TM-transporter), ceramide kinase* and also *dunce* were significantly higher in 5 day-old individuals when compared to newly emerged ones. In queens, up-regulation was denoted for *TM-transporter, ceramide kinase* and downregulation for *paramyosin*.

In a second step we compared the transcript level of each of the ten genes across phenotypes. In these comparisons, we set the focus on diploid males and calculated gene expression differences as fold change through

pairwise REST comparisons of the levels in diploid males (set as 1) against haploid males, workers and queens. In newly emerged haploid males (Table 2), transcript levels were higher for *paramyosin* and *FAS* and lower for *ceramide kinase* and *DGK* when compared to diploid males. Newly emerged workers had lower expression levels for three genes (*TM-transporter, ceramide kinase* and *groucho*) than diploid males, whereas in queens, seven of the ten genes turned out to be over-expressed.

In 5-day-old haploid males (Table 3), expression levels continued to be lower for *ceramide kinase* and *DGK*, and were also lower for *groucho* when compared to diploid males. Workers had lower expression levels for two genes (*dunce* and *paramyosin*) than diploid males. In contast, transcript levels in 5-day-old queens continued to be higher for six out of ten genes, except for *paramyosin*, which was now less expressed than in diploid males of the same age.

The overall picture on gene expression levels for the ten caste-related genes in *M. quadrifasciata* males and females can thus be summarized as follows. Irrespective of ploidy levels in the male sex, two genes, *dunce* and *groucho*, showed similar trends in their regulation with regard to age, with *dunce* being up and *groucho* down-regulated in 5-day-old males. For females, similarities between the castes were noted in directionality of age-related change for *TM-transporter, ceramide kinase* and p*aramyosin*, the first two being up and the latter down-regulated in 5-day-old queens and workers. A second major result is that diploid males are much more similar to haploid males and workers in expression levels of these genes than they are to queens, both at emergence from the brood cells, as well as during the period of sexual maturation, here studied at day five of adult live.

Before entering the discussion, a *caveat* should be brought up at this point. Although not explicitly in the focus of this study we noted that the directionality of caste-specific differences in expression levels differed for some of the genes when compared to the results previously reported by Judice *et al.* (2006). For instance, we found the expression levels for *dunce* and *groucho* to be higher in newly emerged queens than in workers of the same age. We attribute these discrepancies, which obviously have consequences for the interpretation of RT-qPCR results in relation to caste

and division of labor in this social bee, to the use of different reference genes. Whereas Judice *et al.* (2006) relied on *28S rRN*A as reference gene we followed a more stringent recommendation (Vandesompele *et al.*, 2002; Bustin *et al.*, 2009; Derveaux *et al.*, 2010) and used two reference genes, one encoding a cystoplasmatic actin and the other ribosomal protein 49. Furthermore, these two genes had in the meantime been validated as suitable reference genes for RT-qPCR studies in the honey bee (Lourenço *et al.*, 2008). We are thus confident that the comparisons on gene expression levels in relation to age and genotype/phenotype presented herein reflect real differences. Furthermore, by using REST software (Pfaffl *et al.*, 2002) we employed a stringent statistical analysis procedure specifically developed for quantitative gene expression analysis.

Discussion

Diploid males are “misfits” in terms of hymenopteran population genetics (Liebert *et al.*, 2004; van Wilgenburg *et al.*, 2006) and in social species even more so as they are also a burden on colony productivity and reproductive success (Duchateau and Mariën, 1995; Whitehorn *et al.*, 2009). They are nevertheless of interest for investigating gene regulatory networks underlying phenotype differentiation. Gene regulatory network differences have been computationally predicted from gene expression data in honey bee queen and worker larvae (Cristino *et al.*, 2006; Barchuk *et al.*, 2007), but so far none such prediction exists for comparisons between the sexes. A complicating factor herein are the differences in ploidy levels between normal male and female Hymenoptera, and even though gene dosage may be compensated by alternatively silenced paternal or maternal alleles in the female sex, this may not necessarily be the case for all genes.

Among the ten genes for which we quantified expression levels, *TM-transporter*, *paramyosin*, *ceramide kinase*, *DGK*, *dunce* and *groucho* showed significant variation with respect to age and among sex and caste phenotypes. Paramyosin is a structural component of insect flight muscle (Beinbrech *et al*., 1985; Hooper *et al*., 2008). We would, thus, expect its

expression to be related to maturation processes in *Melipona* flight muscle. The observation that its expression was significantly up-regulated in diploid males and slightly so in haploid ones (Figure 1), is in accordance with flight muscle maturation in preparation for mating flights in haploid males (van Veen *et al*., 1997), but it was rather surprising to see this trend as well in diploid ones, as they generally do not leave the colonies. In contrast, the down-regulation in *paramyosin* expression seen in 5-day old queens may reflect their earlier adult maturation. Such precociousness can be inferred from the high locomotor activity of young virgin queens, which soon after emergence must find a safe place within the colony where they can hide to avoid being attacked by workers (Engels and Imperatriz-Fonseca, 1990).

Ceramide kinase mediates the maintenance of ceramide levels, the latter being of importance for the local regulation of phospolipase C (PLC) activity and consequent modulation of intracellular signal transduction by phosphatidylinositol-biphosphate (PIP2). In this context ceramide kinase has been shown to be involved in phototransduction in *Drosophila melanogaster* (Dasgupta et al., 2009). Interestingly, *ceramide kinase* expression was significantly up-regulated in both female castes of *M. quadrifasciata* within the first days after emergence from the brood cells, but not so in haploid or diploid males (Figure 1). Furthermore, ploidy levels may play a role in *ceramide kinase* expression levels, as it was less expressed in haploid males when compared to diploid ones. Ploidy levels may also be associated with *diacyl glycerol kinase* (*DGK*) expression rates, as indicated through comparisons of haploid and diploid males (Tables 2 and 3). In *Drosophila melanogaster*, DGK is produced in specific neurons and is part of a neuronal signal transduction pathway (Harden *et al*., 1993). In honey bees, *DGK* was denoted as more expressed in brains of old foragers than in newly emerged workers (Tsuchimoto *et al*., 2004).

Behavioral modulation is also the primary biological function attributed to the gene *dunce*. It encodes a cyclic AMP phosphodiesterase that is critical for fruit fly neuronal development and learning and memory (Bellen *et al.*, 1987; van Swinderen, 2007). Furthermore, mutations in *dunce* also affect male sexual behavior and *dunce* females are unresponsive to sex peptide (Chapman *et al.*, 1996). As we could show, *dunce* expression is up-regulated

in males and females of *M. quadrifasciata* during early adult life, inferring general maturation processes in behavioral responses. In a previous study, its higher expression in newly emerged workers than in queens (Judice *et al*., 2006) had led us to conclude that it may play a role in cooperative behavior of workers, based on evidence from fruit flies (Tinette *et al.*, 2004). Nevertheless, with the fact in mind that *dunce* was denoted as less expressed in 5-day-old workers than in diploid males (Table 3), a role in cooperative behavior seems less likely, because males of stingless bees were only exceptionally seen to participate in colony maintenance activities (Imperatriz-Fonseca, 1973; van Veen *et al.*, 1997).

Compared to the relatively specific roles of the aforementioned genes, *groucho* is about as multifaceted as its naming patron. It encodes a member of the conserved TLE/GRG family of co-repressors for multiple transcription factors (Jennings and Ish-Horowicz, 2008). In the fruit fly, groucho protein is required in neurogenesis, segmentation and sex determination (Paroush *et al*., 1994). The molecular mechanisms through which Groucho acts to repress transcription are now gradually emerging, one of these being its interaction with a histone deacetylase (Winkler *et al.*, 2010) resulting in chromatin modification. As we could show, *groucho* expression is significantly down-regulated in *M. quadrifasciata* males during the first five days of adult life (Figure 1), and there is a contrasting picture in relation to females, with *groucho* transcript levels being significantly lower in newly emerged workers in comparison to diploid males (Table 2), but they are higher in 5-day-old queens. As it is not a structural gene but a transcriptional modulator, altering *groucho* levels by means of an RNAi protocol should be a feasible strategy to get a glimpse at its role in social bees, especially since epigenetic modification has been shown to be a major factor in honey bee caste development (Kucharski *et al.*, 2008).

From a general perspective, a trend that apparently distinguishes the two sexes in *M. quadrifasciata* is an apparent overall increase in transcript levels in young females, considering that in both queens and workers the median fold change in expression levels were higher than 1 for seven out of the ten genes at day five after emergence from the brood cells (Figure 1). In contrast eight of the ten genes were less expressed in 5-day-old haploid

males and five out of ten in diploid males. Possibly, this difference between sexes may be related to life span, which is much higher in females than in males, not only in *M. quadrifasciata* (Camargo, 1982) but also in honey bees (Ruttner, 1966) and most other social Hymenoptera. Finally, the largest overall difference in gene expression levels was denoted when comparing diploid males to queens (Tables 2 and 3). Since the gene set used herein was derived from subtractive hybridization libraries contrasting queens and workers, which greatly differ in fertility, life span and roles in the colony, it is plausible that the large discrepancy in gene expression levels seen between diploid males and queens is an even further extrapolation of this trend. Such gene sets may thus be suitable for future investigations towards gene networks underlying life history divergence in the castes and sexes of highly social insects.

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Table 1- Real-time PCR primer sequences and their respective target genes

Target genes for qRT-PCR	Primer	Sequence	Source
ribosomal protein 49	rp49F rp49R	5'-CGTCATATGTTGCCAACTGGT-3' 5'-TTGAGCACGTTCAACAATGG-3'	Lourenço *et al.*, 2008
actin	ActMelF actMelR	5'-CGGGTGGTGCGATAATCTTG-3' 5'-GGGTATGGAAGCCTGCGGTATC-3'	Judice *et al.*, 2004
transmembrane transporter	Mq1F Mq1R	5'-TTGCTATCTCGTGCGTCTTG-3' 5'-GCGAACATGCCGAATAAACG-3'	This study
permease	Mq2F Mq2R	5'-CCCGTTAGATGCGACTCAG-3' 5'-CATGCTTGTCCGTTTCATATTG-3'	This study
ceramide kinase	Mq3F Mq3R	5'-CACGTTCGTGGTGAAGAAGA-3' 5'-CCTCGTGTATCCAATCGTCC-3'	This study
Mq5	Mq5F Mq5R	5'-GGATTTCAAAGTGGCTGGC-3' 5'-GTTAATCACGTTCAATCGCCC-3'	This study
dunce	DunF DunR	5′-AGCCGACCTGCGACTTCTC-3′ 5′-ACATGGACATTAGCCCAATGTG-3′	Judice *et al.*, 2006
amino acid-polyamine transporter	AmitranspF AmitranspR	5′-AGGGAAGATCCCGTCAAGAA-3′ 5′-GGGTCGTGTAAAATGCCATGT-3′	Judice *et al.*, 2006
paramyosin	PmyosF PmyosR	5′-ATCCGAGGGAAGATCCAGGTA-3′ 5′-TGCCTCTTGTAGATGCTCATTTTC-3′	Judice *et al.*, 2006
diacylglycerol kinase	DGKF DGKR	5′-CTTCGTATCGATGCCAGCAA-3' 5′-TTTTGTTGTTCGTCAATCCGTTT-3′	Judice *et al.*, 2006
fatty acid synthase	FASF FASR	5′-GATCGCGGGATTGATACCTACT-3′ 5′-TCGACGGTAACAAAAGTCAAGGA-3′	Judice *et al.*, 2006
groucho	GrouF GrouR	5′-CGGCGGACGGTTCGA-3' 5′-GATCCCACGAACGCACTGT-3′	Judice *et al.*, 2006

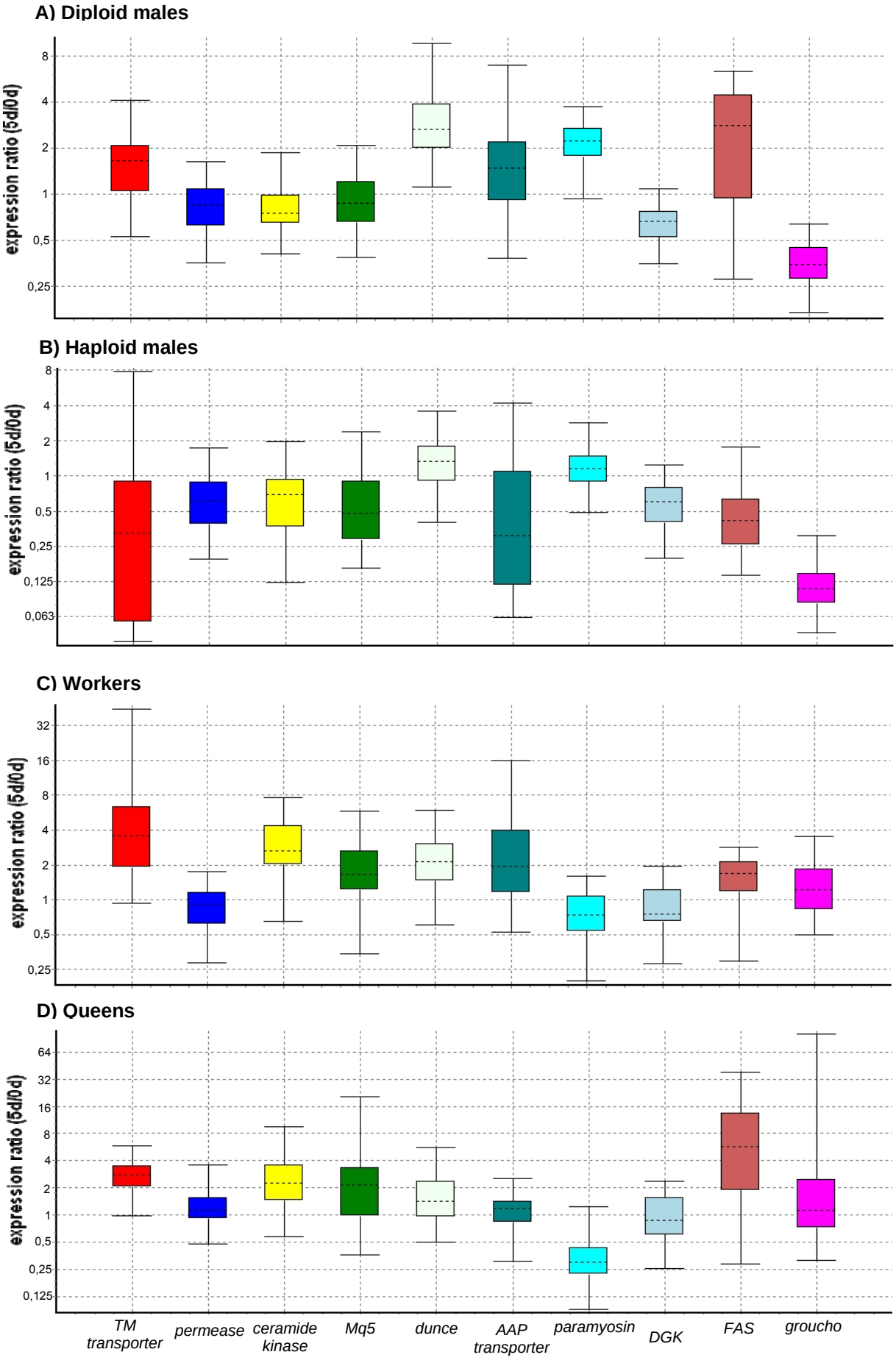
Table 2 - Pairwise comparisons for gene expression levels in newly emerged diploid males against haploid males, workers and queens. Fold-change and direction are given based on REST (Pfaffl *et al.*, 2002) results. Only statistically significant differences ($P \leq 0.05$) are shown

gene	haploid male	worker	queen
transmembrane transporter		3,01x down	1,64x up
permease			2,42x up
ceramide kinase	2,08x down	2,86x down	
Mq5			2,31x up
dunce			2,90x up
amino acid –polyamine transporter			2,67x up
paramyosin	2,19x up		2,16x up
diacylglycerol kinase	2,05x down		
fatty acid synthase	2,01x up		2,38 x up
groucho		4,34x down	

Table 3 - Pairwise comparisons for gene expression levels in 5-day-old diploid males against haploid males, workers and queens. Fold-change and direction are given based on REST (Pfaffl *et al.*, 2002) results. Only statistically significant differences ($P \leq 0.05$) are shown

gene	haploid male	worker	queen
transmembrane transporter			2,85x up
permease			3,59x up
ceramide kinase	2,84x down		1,91x up
Mq5			5,65x up
dunce		1,95x down	
amino acid –polyamine transporter			
paramyosin		1,82x down	3,17x down
diacylglycerol kinase	2,37x down		
fatty acid synthase			5,13x up
groucho	1,99x down		4,61x up

Figure 1- Changes in gene expression levels during early adult life of *Melipona quadrifasciata* diploid males (A), haploid males (B), workers (C) and queens (D). Fold change values refer to transcript levels detected in five-day old bees in comparison to newly emerged ones (the latter set as equal to 1). Box-and-whisker plots show median (dotted line), upper and lower quartiles (boxed), upper maximum and lower minimum for each gene. Asterisks indicate significant differences (REST randomization significance test, $P \leq 0.05$).


A) Diploid males
expression ratio (5d/0d)
8
4
2
1
0,5
0,25
B) Haploid males
expression ratio (5d/0d)
8
4
2
1
0,5
0,25
0,125
0,063
C) Workers
expression ratio (5d/0d)
32
16
8
4
2
1
0,5
0,25
D) Queens
expression ratio (5d/0d)
64
32
16
8
4
2
1
0,5
0,25
0,125
TM transporter
permease
ceramide kinase
Mq5
dunce
AAP transporter
paramyosin
DGK
FAS
groucho

CAPÍTULO 2

Differential gene expression in haploid versus diploid *Melipona quadrifasciata* (Hymenoptera: Apidae) males*

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Running title: Gene expression in *Melipona* males

Key words: stingless bee; diploid male; subtractive hybridization; differential gene expression; real time PCR.

* Artigo escrito de acordo com as normas da revista Insect Molecular Biology.

Abstract

In most Hymenoptera, apart from females (workers and queens) produced from fertilized eggs and haploid males from unfertilized eggs, males larvae can also develop from fertilized eggs since they are homozygous at sex locus. Haploid and diploid males of *Melipona quadrifasciata* are morphologically similar, making it difficult to distinguish by visual inspection within the colony. However, differences in life span, sexual maturity and length of the testes of these males have been detected. To verify whether these differences might be associated with differential gene expression we generated 443 high quality ESTs using Representational Difference Analysis of cDNA (cDNA-RDA). Biological functions ontology of differentially expressed genes between males revealed that haploid males expressed genes involved in stress responsiveness, biosynthetic process, reproductive processes and spermatogenesis, whereas the majority of genes from the diploid male library was involved in cellular organization, nervous system development and regulation of gene expression. Quantitative PCR analyses confirmed the differential expression of ten of seventeen candidate genes. Further analysis with 5 days-old males revealed changes in transcript abundance in this period in which the sexual maturation occurs and the sperm begin to migrate from the testes to the seminal vesicle.

Introduction

While vital to sexual reproduction, the investment in males generates a twofold conflict in the haplodiploid social insects, including bees, wasps and ants. Whereas on the one hand the production of males takes resources from the investment in colony size, which is essentially defined by the size of its worker population, it also is a source of reproductive conflict between the queen and her worker daughters (Velthuis *et al*., 2005; Ratnieks *et al*., 2006).

In the honey bee, *Apis mellifera*, the sole function attributed to males is to mate with new queens, while the males of stingless bees may also carry out some within-hive activities that in *Apis* are exclusively performed by workers, such as nectar dehydration, wax manipulation and trophallaxis (Imperatriz-Fonseca, 1973; Cortopassi-Laurino, 1979; Kerr, 1997; van Veen *et al*., 1997; Nogueira-Neto, 1997).

Male larvae normally develop from unfertilized eggs, but in more than 60 species of Hymenoptera, including sawflies, parasitoid wasps and ants, bees and wasps, diploid males have been found. These males develop from fertilized eggs that are homozygous at the sex determining locus (Stouthamer *et al*., 1992; Cook, 1993; Cook & Crozier, 1995; Butcher *et al*., 2000; van Wilgenburg *et al*., 2006; de Boer *et al*., 2007). Diploid males are usually sterile or not viable at all (Woyke & Skowronek, 1974; Petters & Mettus, 1980; Godfray & Cook, 1997). Their production, therefore, is highly disadvantageous, representing a significant loss in the potential worker production, thus imposing an impediment to colony growth and survival (Ratnieks, 1990; Roubik *et al*., 1996; Zayed *et al*., 2003; Herzner *et al*., 2006; Armitage *et al*., 2010).

In order to avoid the disadvantageous presence of these males in the colony, honey bee workers recognize and eliminate the diploid males at a very early developmental stage (Woyke, 1963; Santomauro *et al*., 2004). This is possible because honey bee workers have access to brood cells and constantly feed them. In contrast, diploid males of stingless bees, including those of *Melipona quadrifasciata*, are viable and can reach adulthood but have a less lifespan (Camargo, 1979). When their presence is detected by workers, these frequently eliminate the queen, who produces these males,

and rear a replacement, thus avoiding the death of the colony (Ratnieks, 1990).

Although not easily distinguishable from haploid males by visual inspection, a meticulous study on *M. quadrifasciata* males showed that diploid males have a lower average life expectancy than haploid ones and females (Camargo, 1982). Furthermore, diploid males produce less spermatozoa, and these show a delay in the migration from the testes to the seminal vesicles, which may explain their delayed sexual maturity (Camargo, 1984). Morphologically, pupae of diploid males of *M. quadrifasciata* have shorter testes than haploid ones, as well as a wider thorax (Tavares *et al*., 2003), these being characteristics also found in diploid males of other Hymenoptera (Woyke, 1974, 1978; Ross & Fletcher, 1985; Naito & Suzuki, 1991; El Agoze & Periquet, 1991, Duchateau & Marien, 1995).

As such differences between diploid and haploid males cannot simply be a result of differences in their ploidy levels, and because allelic combinations at the sex locus are only the initial step in sex determination, we expected that a number of genes should turn out to be differentially expressed, leading to the differences in morphology, longevity, sexual maturity, as well as other features not yet studied in the two types of males.

Studies on differential gene expression are making an important contribution to our understanding of caste development in social insects in the area of gene expression, especially so in the honey bee (Evans & Wheeler, 1999; Corona *et al.*, 1999; Hepperle & Hartfelder, 2001; Barchuk *et al*., 2007), but also in the stingless bee *M. quadrifasciata* (Judice *et al*., 2006). So far, however, no efforts have been directed at elucidating gene expression differences in the development of the male sex of highly eusocial bees. Not easily being conceivable in honey bees, where obtaining diploid males is complicated by their removal by workers at a very early larval stage, we investigated this question in the stingless bee *M. quadrifasciata.* The molecular basis characterizing and setting apart diploid from haploid males is an interesting question not only for the male sex, but also for the understanding caste development in the female one of social Hymenoptera. Whereas female caste differentiation is environmentally and epigenetically driven in most species (Hartfelder & Emlen, 2005; Kucharski *et al*., 2008),

and also includes a genetic component in some species, such as *M. quadrifasciata* (Kerr, 1950), the males differ primarily in their ploidy levels, those posing an interesting problem in developmental biology of social Hymenoptera.

To investigate the molecular underpinnings setting apart haploid from diploid males of *M. quadrifasciata*, we employed a Representational Difference Analysis of cDNA (cDNA-RDA) technique (Pastorian *et al*., 2000), a suppression subtractive hybridization strategy which allows enrichment of differentially expressed genes and removal of common ones. After cloning and sequencing ESTs representing differentially expressed sequences we measured the transcript abundance of a set of these in newly emerged and 5 day-old males. The latter represent the age class at which sexual maturation occurs and sperm begins to migrate from the testes to the seminal vesicle (Camargo, 1984). This quantitative analysis of transcript levels of a specific gene set will gave us additional information on the temporal expression of candidate genes for sexual differentiation.

Results

RDA library sequences and bioinformatics analyses

We successfully constructed two subtractive cDNA libraries that resulted in a total of 528 sequenced clones, of which 443 were of appropriate quality (Phred quality ≥ 20). The set of 443 good quality reads was comprised of 207 ESTs from the diploid male library and 236 ESTs from that of haploid males. Subsequent CAP3 clusters compressed these into 86 unique sequences, composed of of 18 contigs and 25 singlets that originated from the diploid male RDA library, and 22 contigs and 21 singlets from the haploid male library.

The sequence analysis using blastn and blastx algorithms revealed significant GenBank matches (E-scores $< e^{-5}$) for 28 unique sequences of the haploid male library (Table IS - Supplementary Material). For the diploid male library, 31 unique sequences showed significant matches to genes deposited in GenBank (Table IIS - Supplementary Material). Overall, the ESTs

sequenced from haploid male RDA library showed similarity with sequences of *Drosophila* (32.5%), *Apis* (25.6%) and stingless bees (7%), especially *M. quadrifasciata anthidioides* and *M. bicolor* (Figure 1A). In the diploid male library, the percentage of ESTs with similarity to *Drosophila* and *Apis* sequences were slightly higher, being 41.8% and 30.2%, respectively (Figure 1B). Both subtracted libraries also presented considerable proportions of ESTs without significant match to sequences in the nr database (34.9% for the haploid and 28% for the diploid male libraries).

When analyzing the ESTs for Gene Ontology attributes of biological process we noted that for haploid males a relatively higher number of the differentially expressed genes was associated with stress responsiveness, biosynthetic processes, reproductive processes, and spermatogenesis, whereas for the diploid male library, the majority of genes was involved in cellular organization, nervous system development and regulation of expression (Figure 2).

RT-qPCR analysis of RDA library genes - comparing newly emerged haploid and diploid males

For this analysis we selected a set of the unique sequences from the haploid and diploid male libraries. The primary criteria for composing this set were that it should include (i) genes that were particularly well represented in the two subtractive libraries; (ii) genes representing a broad spectrum of GO biological processes, and (iii) genes of unknown function or no-match sequences. The gene-specific primers designed for 17 of these genes reliably amplified specific products of predicted product length (Table IIIS - Supplementary Material).

In order to confirm their differential expression we initially compared newly emerged haploid and diploid males. The expression ratio for each gene is shown as a box-and-whisker plot denoting fold change in newly emerged diploid males relative to haploid ones (expression levels in haploid drones were set equal to 1). For the seven genes selected from the haploid male library, the relative quantification of transcript levels showed that the

genes coding for *myosin regulatory light chain 2*, *troponin T* and *rieske iron-sulfur protein* were significantly more expressed in haploid males when compared with diploid males (REST, $P \leq 0.05$). Only *serine protease inhibitor 5* of the haploid library did not confirm to the expected higher expression in these samples (Figure 3).

Regarding the ten genes of the diploid male RDA library, three genes showed significantly higher expression ratios in newly emerged diploid males: a *calcium-transporting ATPase* encoding gene, the CG8785 gene of unknown function, and T2n1, this being a no-match sequence. Significantly higher expression ratios in haploid males were denoted for *CG32464*, *immunoglobulin like-fibronectin type III* and T2n8 (Figure 3).

In a second step we set the focus on an expression analysis of these same genes during the period of sexual maturation, comparing transcript levels in newly emerged drones with those in 5 day-old ones. Notably, for diploid males (Table 1), all the genes selected for analysis from the haploid male RDA library were less expressed in 5 day-old males when compared to newly emerged ones ($P \leq 0.05$). Yet, for the genes selected from the diploid male RDA library the trend was opposite, except for T2n1 and T2n3, which were down-regulated. Interestingly, the same picture was denoted for 5 day-old haploid males, except that all the genes identified in diploid male library were up-regulated in 5 day-old haploid males (Table 2).

Discussion

In this study we identified several different products between diploid and haploid males of *M. quadrifasciata* using the cDNA-RDA approach (Hubank & Schatz, 1994). The standard cDNA-RDA protocol was ideally suited to rapidly reduce the number of candidate genes in a specific manner, thus allowing the identification of 43 unique sequences for each subtracted library constructed. Despite the lack of sequence information and functional characterization of *M. quadrifasciata* genes, BLAST analysis revealed that most of the identified sequences, in both subtracted libraries, presented

homology with *Drosophila* and *Apis* sequences (Table IS and Table IIS-Supplementary Material). This confirmed the high level of conservation between the genes of these insects.

Even with the considerable advance in genomics and transcriptome studies, many ESTs presented no significant homology to any sequence available in the database as well as no functional results in Gene Ontology. Three genes no-match to sequences in the nr database (T2n1, T2n3 and T2n8) were quantified by qRT-PCR and represented diploid males differentially expressed genes. These results may indicate that we are faced with new and possibly specific genes for stingless bees.

Transcripts of three muscle structural genes were detected in the haploid male library. The *myosin regulatory light chain 2* (*Dmcl2*) gene, represented by 66 ESTs in haploid male library, encodes a protein that acts as physiological substrate for phosphorylation by myosin light chain kinase triggering the contraction of muscle cells and certain processes in nonmuscle cells (Tohtong *et al.*, 1995). The gene encoding troponin T, a protein that acts in the regulation of muscle contraction, in turn, is a key component coordinating all the components of the troponin-tropomyosin complex (Perry, 1998), while the *actin 88E* gene of *Drosophila* encodes an actin isoform expressed in the indirect flight muscle (Fyrberg *et al.*, 1983). The differential expression of these genes, except for *actin 88E*, was confirmed by RT-qPCR in newly emerged and in 5 day-old haploid males, allowing to infer that the presence of these transcripts may be associated to the flight capability of males during nuptial flight.

In addition, we identified three homologs of structural constituent of ribosome genes as haploid male-expressed: *40S ribosomal protein*, *ribosomal protein P2* and *ribosomal protein S16* genes. The first gene is alternatively referred as *stubarista*, and encodes a ribosome-associated protein proposed to play a positive role in translation and cell size as well as a negative role in cell proliferation (Melnick *et al*., 1993). *40S ribosomal protein* differential expression was tested by real time PCR and revealed no significant differences between newly emerged diploid and haploid males (Figure 3). However, 5 day-old males presented lower expression levels than newly emerged males (Tables 1 and 2).

The r*ieske iron-sulfur protein* gene was also identified in the library of haploid males. Based on the *Drosophila* homolog it is predicted to be involved in the mitochondrial electron transport, ubiquinol to cytochrome c, acting as the initial electron acceptor in the rate-limiting step of the catalytic reaction (Berry *et al*., 2000). Significant differences in *rieske iron-sulfur protein* expression were detected in the newly emerged males, being less expressed in diploid males than in haploid ones. Considering the 5 day-old samples, we observed lower levels of expression in diploids than in haploids.

The presence of the protein kinase 61C encoding gene in haploid male library is an interesting finding. Protein kinases compose a family of serine/threonine kinases involved in the regulation of a wide range of cellular responses, including, cell proliferation, differentiation and survival (Su & Karin, 1996). Additionally, according to the Gene Ontology analysis, this gene is involved with reproductive process, sex differentiation and spermatogenesis. The absence of *protein kinase 61C* transcripts in newly emerged diploid males may be related to the sexual maturation delay observed in these males. Camargo (1984) observed that diploid males of *M. quadrifasciata* had a lower amount of spermatozoa in the testis and their migration to the seminal vesicles began on the ninth day. On the other hand, in haploid males the percentage of migration is higher and began earlier, on the seventh day.

We detected *ecdysteroid-regulated gene E74* and *serine protease* transcripts in haploid and diploid male libraries. The presence of these transcripts in both libraries may be isoforms of alternative splicing. Since the genome of *Melipona* is not sequenced, these results could be verified by mapping of EST on the genome of *Apis* to obtain positional information of the ESTs.

Ecdysteroid-regulated genes of dipteran insects are involved in oogenesis (Cho *et al*., 1995; Kozlova & Thummel, 2000; Sun *et al*., 2002). No significant *ecdysteroid-regulated gene E74* expression was detected in the adult honey bee males or worker abdomens. Its expression was detected selectively in ovary of queens and preferentially in the brain of the adult workers (Paul *et al*., 2005). Significant differences in the *ecdysteroid-regulated gene E74* expression of 5 day-old diploid males suggest that this

gene may be involved in other regulatory pathways besides those related to reproductive processes and neural functions.

The *serine protease* gene, a *D. melanogaster* homolog involved in negative regulation of Toll signaling pathway, was identified in both male libraries. This pathway is required for the establishment of the dorsal-ventral axis in *Drosophila* embryos (Hashimoto *et al*., 1988; Kambris *et al*., 2002; Moussian, 2005) and plays an important role in larval and adult responsiveness to microbial infections (Lemaitre *et al*., 1996; Anderson, 2000; Ferrandon *et al*., 2007). Toll-like receptors are considered to be the primary sensors of invading pathogens, recognizing conserved microbial molecules and activating signaling pathways that are pivotal to insect humoral immune responses (Medzhitov, 2001; Takeda & Akira, 2005; Leulier & Lemaitre, 2008). The presence of these transcripts represented by different numbers of ESTs in the haploid and diploid male libraries (7 and 31, respectively) may indicate that the defense mechanisms against pathogens are more early activated in diploid males than in haploid ones soon after its emergence, what was confirmed in qRT-PCR analysis.

Despite the fact that the cDNA library used in this study has been prepared from newly emerged mRNAs, we found that the majority of the transcripts were still expressed after five days of emergence indicating that these genes are important throughout adult development. This study represents an important first step to understanding patterns of gene expression of haploid and diploid males, providing molecular data related to the processes of differentiation of males of a stingless bee.

Experimental procedures

Bees and messenger and total RNA extraction

Males of *Melipona quadrifasciata* were obtained from colonies kept at the meliponary of the Federal University of Viçosa (Viçosa – MG, Brazil). Haploid males were collected from combs taken from non-inbred colonies, whereas diploid males were obtained from inbred colonies produced from a brother-sister mating protocol (Camargo, 1974, 1979). Ploidy was confirmed by cytogenetic analysis according to Imai *et al*. (1988).

Brood combs containing late pupal stages were removed from the colonies and kept in an incubator at 28 ^{o}C. Newly emerged bees were collected within 4 to 6 h after emergence from the brood cells. In order to assess transcript abundance of differentially expressed genes in the libraries of zero days, newly emerged bees were kept in Petri dishes in an incubator (28^{o}C) with *ad libitum* access to sugar syrup (50%) and fermented pollen (Camargo, 1979) until they reached 5 day-old. All samples were frozen in liquid nitrogen and stored at -80^{o}C until RNA isolation.

Total RNA of whole-body extracts of two newly emerged and 5 day-old males was extracted using 1 mL of TRIzol (Invitrogen) following the manufacturer's protocol and treated with 0.1 U DNAse I (Invitrogen) to eliminate potential genomic DNA contamination. RNA purity and integrity were checked by agarose gel electrophoresis under denaturing conditions and quantified spectrophotometrically (Nanovue, GE Healthcare). All RNA samples (five for each age class) were stored at -80ºC until molecular analysis.

Representational Difference Analysis (RDA)

Total RNA extracted from newly emerged haploid and diploid males using TRIzol reagent (Invitrogen) was purified to obtain mRNA profiles by means of an Oligotex Total RNA kit (Qiagen). From these, 5 µg of each mRNA sample was reverse transcribed for first strand synthesis and then subjected to a long distance PCR protocol employing the SMART PCR cDNA synthesis kit (Clontech) to obtain double-stranded cDNA profiles.

Subtractive hybridizations were performed following a cDNA-RDA protocol (Pastorian *et al*., 2000), with modifications for application in bees (Judice *et al*., 2006). Double-stranded cDNA obtained by long distance RT-PCR (1 µg) were digested with *Mbo*I restriction enzyme (Fermentas Life Sciences), ligated to the set of R-adapters (Table IIIS – Supplementary Material), and amplified by PCR according to Hubank and Schatz (2000), in order to generate cDNA representations for newly emerged haploid and diploid males. Enrichment of differentially expressed transcripts was achieved in two successive rounds of PCR amplifications employing different

adapters (J and N: Table IIIS – Supplementary Material) and sequential subtractive hybridizations of the tester to an excess of driver cDNA, first in a 1:100 and then in a 1:800 ratio (Hubank & Schatz, 2000). The two rounds of subtractive hybridizations removed transcripts shared between the driver and tester populations, thus enriching the libraries for differentially expressed transcripts. After each of these successive steps, cDNAs were purified using Illustra GFX PCR purification kit (GE Healthcare). We generated two RDA libraries, one for haploid males (tester), subtracted against diploid males (driver), and the other where diploid male cDNA was the tester and haploid male cDNA the driver population.

These differential products were cloned into the pGEM-T Easy Vector System (Promega) and used to transform chemically competent *E. coli* DH5α cells. The plasmid inserts were purified by a miniprep protocol and sequenced in an ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems) using a BigDye Terminator version 3.0 Cycle Sequencing Reaction (Applied Biosystems) with an M13 forward primer.

Sequence analysis

Sequencing reads were passed through an E-Gene annotation pipeline (Durham *et al*., 2005). In its first step the pipeline was designed to detect and remove ribosomal RNAs and mitochondrial DNA. Subsequently, vector sequences were trimmed by means of Crossmatch. The quality of sequences was analyzed by the Phred-Phrap program module and those sequences presenting appropriate quality (Phred quality $\geq$ 20) were submitted to assembly by the CAP3 software (Huang & Madan, 1999) in order to obtain unique sequences. These were then entered as queries into BLAST searches (blastx and blastn) against sequences deposited in a non-redundant (nr) database (GenBank).

The GeneOntology database (Ashburner *et al.*, 2000) was used to infer the possible biological function of ESTs that presented similarities to genes of known function in other organisms. GeneOntology (GO) terms attributed to their respective *Drosophila* orthologs were retrieved from the Flybase server (http://flybase.org).

Validation of differential expression

Differential expression was further validated by real-time quantitative RT-PCR (RT-qPCR) for a set of ten genes selected from the diploid and seven genes from the haploid male RDA library. For these we designed gene-specific primers using Gene Runner version 3.05 and Primer3 (http://frodo.wi.mit.edu/primer3) softwares. The amounts of gene-specific transcripts in each sample were normalized against two control genes, one encoding ribosomal protein 49 (*rp49)* and one for a cytoplasmatic actin (*act*). Primers serving as RDA adapter and those targeting specific genes, as well as expected amplicon length are listed in Table IIIS (Supplementary Material).

For comparability between assays, primer efficiencies were obtained by quantitative PCR on serially diluted sample standards. The slope of the curve was used according to Pfaffl (2001) to calculate amplification efficiency of the respective primers as: efficiency = $10(^{-1/slope})$.

DNAse I (Invitrogen) treated total RNA samples from newly emerged and 5 day-old haploid and diploid males were used as templates for first-strand cDNA synthesis using Superscript II (Invitrogen) enzyme and oligo $(dT)_{18}$ primer (Fermentas Life Sciences).

Real-time PCR amplification cycles were done in an ABI Prism 7500 system (Applied Biosystems) and run with each sample being in quintuplicates (five biological replicates for each male phenotype and age class). The total reaction volume of 14 µL was comprised of 7 µL of SYBR Green (Applied Biosystems), 1 µL single-strand cDNA template (diluted 1:10), 0.8 µL of each of gene-specific forward and reverse primer (10 pmol/µl) and 4.4 µL of deionized water (MiliQ, Milipore). PCR conditions were 50 ºC for 2 min and 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. The specificity of the amplified products was monitored by dissociation curve. Each sample was assayed in technical triplicates, and for each experiment a non-template reaction was included as negative control. Two inter-run calibrators were run on each plate to control for interplate variation, as suggested in the MIQE recommendations (Bustin *et al.*, 2009).

The expression levels for the genes of interest in each sample were given as the means of the respective technical triplicates after subtracting inter-run differences. Relative expression levels were calculated from these using the comparative Ct method and expressed as $2^{-\Delta\Delta Ct}$ (Pfaffl, 2001). For statistical analysis of expression differences, the original Ct values were used as input into randomization tests implemented in the Relative Expression Software Tool, REST (Pfaffl *et al*., 2002), considering values of $P \leq 0.05$ as statistically significant.

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Table 1- Pairwise comparisons for gene expression levels in 5 day-old diploid males against newly emerged diploid ones. Fold-change and direction were calculated by REST analysis (Pfaffl et al., 2002). Only statistically significant differences ($P \leq 0.05$) are shown

Genes	Fold change in expression for 5 day-old diploid males
Genes identified in haploid males	
myosin regulatory light chain 2	2,21 x down
actin 88E	3,38 x down
40S ribosomal protein	3,48 x down
troponin T	4,00 x down
serine protease inhibitor 5	3,81 x down
ecdysteroid-regulated gene E74	4,36 x down
rieske iron-sulfur protein	2,15 x down
Genes identified in diploid males	
T2n1 - No match	11,11 x down
CG32464-PK	2,49 x up
T2n3 - No match	11,90 x down
CG8785-PA	2,96 x up
calcium-transporting ATPase	4,49x up
T2n8 - No match	4,39 x up
absent, small, or homeotic discs 2	7,39 x up
CG8026-PA	7,89 x up

Table 2- Pairwise comparisons for gene expression levels in 5 day-old haploid males against newly emerged haploid ones. Fold-change and direction were calculated by REST analysis (Pfaffl et al., 2002). Only statistically significant differences ($P \leq 0.05$) are shown

Genes	Fold change in expression for 5 day-old haploid males
Genes identified in haploid males	
myosin regulatory light chain 2	2,11 x down
actin 88E	3,37 x down
40S ribosomal protein	2,46 x down
serine protease inhibitor 5	6,21 x down
Genes identified in diploid males	
calcium-transporting ATPase	3,50 x up
T2n8 - No match	2,84 x up
absent, small, or homeotic discs 2	5,14 x up

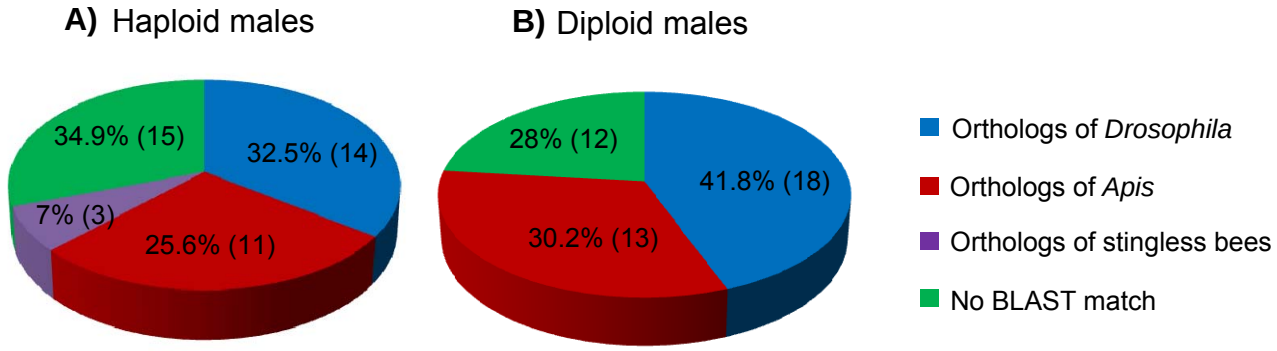

Figure 1- Similartity analysis (blastx) results for unique sequences (contigs and singlets) obtained by sequencing clones of the RDA libraries for haploid (A) and diploid males (B) of *Melipona quadrifasciata*.

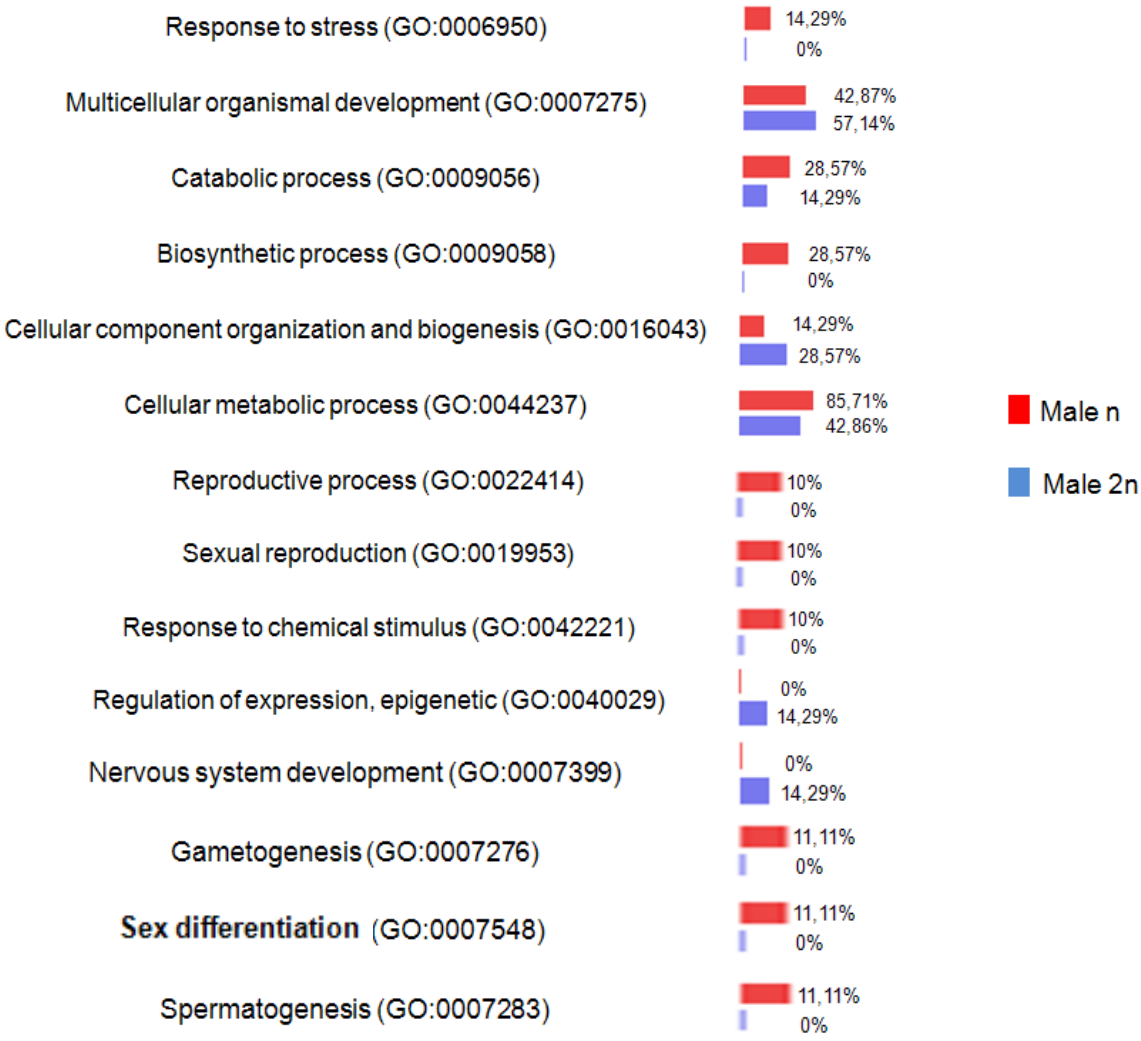

Figure 2- Distribution relative of differentially gene express from haploid (n) and diploid (2n) male RDA libraries according to Gene Ontology (GO) biological functions. GO reference numbers were retrieved from Flybase.

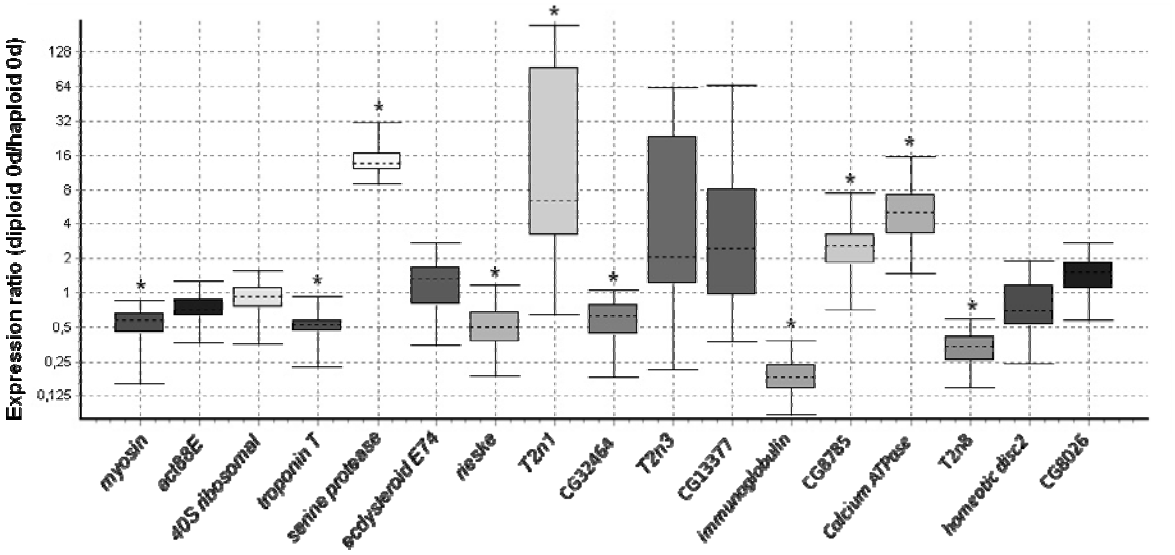

Figure 3- Relative quantification of the expression of 17 transcripts in newly emerged haploid and diploid males of *Melipona quadrifasciata* obtained by RDA. Whisker plots represent the fold change of gene expression in diploid males versus haploid ones. The dotted line in these boxes represents the median and the horizontal bars the minima and maxima of fold change. The line at level 1 of the Y-axis corresponds to the expression level in newly emerged haploid males. Asterisks indicate significant differences (REST, $P \leq 0.05$).

Table IS - Supplementary Material

Table IS - ESTs sequenced from the Representational Difference Analysis (RDA) library of haploid male of *Melipona quadrifasciata*. The respective predicted genes or orthologs retrieved by blastx searches in GenBank were followed by the number of ESTs after assembly (#EST) showing size in base pairs, full name gene and the respective organism and their similarity indices (e-value) and Gene Ontology terms. The ESTs analyzed by RT-qPCR are highlighted in bold.

# EST	Size (pb)	Full name gene (Organism)	e-value	Molecular function or putative biological process
66	**282**	**myosin regulatory light chain 2 (*A. mellifera*)**	**$2e^{-66}$**	**calcium ion binding**
27	**187**	**troponin T (*A. mellifera*)**	**$5e^{-12}$**	**sarcomere organization**
18	**238**	**40S ribosomal protein (*D. melanogaster*)**	**$7e^{-67}$**	**structural constituent of ribosome mitotic spindle elongation, mitotic spindle organization**
12	355	cytochrome c oxidase subunit I (*M. bicolor*)	$9e^{-154}$	cytochrome-c oxidase activity
11	211	cytochrome oxidase subunit I, mitochondrial (*M. quadrifasciata anthidioides*)	$9e^{-97}$	cytochrome-c oxidase activity
8	314	NADH dehydrogenase (*M. bicolor*)	$1e^{-119}$	NADH dehydrogenase (ubiquinone) activity
7	**274**	**actin-88E (*D. melanogaster*)**	**$3e^{-97}$**	**structural constituent of cytoskeleton skeletal myofibril assembly, phagocytosis.**
7	**179**	**serine protease inhibitor 5 (*D. melanogaster*)**	**$2e^{-21}$**	**serine-type endopeptidase inhibitor activity, negative regulation of Toll signaling pathway**
5	**280**	**rieske iron-sulfur protein (*A. mellifera*)**	**$5e^{-56}$**	**mitochondrial electron transport from ubiquinol to cytochrome-c.**
4	134	CG8785 (*D. melanogaster*)	$4e^{-13}$	amino acid transmembrane transporter
2	**409**	**ecdysteroid-regulated E74 (*D. melanogaster*)**	**$9e^{-173}$**	**specific RNA polymerase II transcription factor activity, regulation of biological process, autophagy, post-embryonic development, cell differentiation, regulation of developmental process, larval or pupal development, transcription, programmed cell death**
2	273	CG8026 (*D. melanogaster*)	$2e^{-54}$	transmembrane transporter activity, folic acid transporter
2	159	ribosome-releasing factor 2 (*A. mellifera*)	$6e^{-11}$	GTPase activity
2	150	similar to DumPY : shorter than wild-type family member (*A. mellifera*)	$1e^{-07}$	serine-type endopeptidase inhibitor activity
1	271	heat shock protein 70Cb (*D. melanogaster*)	$3e^{-77}$	chaperone binding

1	219	neutral ceramidase (*D. melanogaster*)	$5e^{-50}$	ceramidase activity, synaptic transmission, sphingolipid metabolic process, photoreceptor cell maintenance, ceramide catabolic process, synaptic vesicle fusion to presynaptic membrane, hatching behavior, synaptic vesicle exocytosis
1	219	ribosomal protein P2 (*A. mellifera*)	$1e^{-57}$	structural constituent of ribosome
1	199	pyruvate kinase (*D. melanogaster*)	$7e^{-48}$	pyruvate kinase activity, phosphorylation
1	194	calcium-transporting ATPase (*A. mellifera*)	$7e^{-35}$	ATPase activity
1	185	ribosomal protein S16 (*D. melanogaster*)	$7e^{-67}$	structural constituent of ribosome mitotic spindle elongation, mitotic spindle organization
1	129	Unc-89 protein (*D. melanogaster*)	$6e^{-36}$	ATP binding, protein serine/threonine kinase activity, adult somatic muscle development, sarcomere organization
1	103	kinesin F (*A. mellifera*)	$5e^{-18}$	microtubule motor activity
1	102	protein kinase 61C (*D. melanogaster*)	$4e^{-19}$	protein serine/threonine kinase activity apoptosis, regulation of organ growth, response to DNA damage stimulus, regulation of cell growth, protein amino acid phosphorylation, cell surface receptor linked signaling pathway, sexual reproduction, reproductive process, organ growth, signal transduction, sex differentiation, spermatogenesis
1	95	aconitate hydratase (*D. melanogaster)*	$1e^{-06}$	aconitate hydratase activity, cofactor metabolic process, generation of precursor metabolites and energy, cellular respiration
1	82	peroxidase (*D. melanogaster*)	$1e^{-13}$	peroxidase activity phagocytosis, response to chemical stimulus, response to stress, catabolic process, oxygen and reactive oxygen species metabolic
5	186	hypothetical protein LOC727105 (*A. mellifera*)	$3e^{-26}$	
1	344	isotig08937 (*A. mellifera*)	$2e^{-28}$	
1	384	isotig21870 (*A. mellifera*)	$2e^{-11}$	
14	277	No-match		
5	216	No-match		
5	192	No-match		
4	219	No-match		
3	199	No-match		
3	219	No-match		
3	216	No-match		

1	341	No-match		
1	282	No-match		
1	245	No-match		
1	231	No-match		
1	226	No-match		
1	214	No-match		
1	200	No-match		
1	102	No-match		

Table IIS - Supplementary Material

Table IIS - ESTs sequenced from the Representational Difference Analysis (RDA) library of diploid male of *Melipona quadrifasciata*. The respective predicted genes or orthologs retrieved by blastx searches in GenBank were followed by the number of ESTs after assembly (#EST) showing size in base pairs, full name gene and the respective organism and their similarity indices (e-value) and Gene Ontology terms. The ESTs analyzed by RT-qPCR are highlighted in bold.

# EST	Size (pb)	Full name gene (Organism)	e-value	Molecular function or putative biological process
31	179	serine protease inhibitor 5 (*D. melanogaster*)	$2e^{-21}$	serine-type endopeptidase inhibitor activity, negative regulation of Toll signaling pathway
12	**219**	**CG32464 (*D. melanogaster*)**	**$1e^{-04}$**	**cell wall macromolecule catabolic process**
10	**223**	**CG13377 (*D. melanogaster*)**	**$4e^{-48}$**	**3-hydroxybutyrate dehydrogenase activity, inter-male aggressive behavior, olfactory behavior, mushroom body development**
6	311	serine protease homolog 54 (*A. mellifera*)	$3e^{-55}$	serine-type peptidase activity
5	**270**	**calcium-transporting ATPase (*A. mellifera*)**	**$3e^{-113}$**	**ATPase activity**
4	182	ribosome-releasing, mitochondrial (*A. mellifera*)	$6e^{-14}$	GTPase activity
2	**304**	**CG8785 (*D. melanogaster*)**	**$1e^{-28}$**	**amino acid transmembrane transporter**
2	204	heat shock protein 70Cb (*D. melanogaster*)	$1e^{-67}$	chaperone binding
2	194	CG31523 (*D. melanogaster*)	$8e^{-70}$	fatty acid biosynthetic process, very long-chain fatty acid metabolic process.
2	**142**	**immunoglobulin-like and fibronectin type III (*D. melanogaster*)**	**$3e^{-30}$**	**structural constituent of muscle, sarcomere organization, mesoderm development**
1	338	hexosaminidase 2 (*A. mellifera*)	$2e^{-46}$	beta-N-acetylhexosaminidase activity
1	297	DAZ associated protein 2 (*A. mellifera*)	$2e^{-77}$	protein binding
1	**266**	**CG8026 (*D. melanogaster*)**	**$4e^{-54}$**	**transmembrane transporter, folic acid transporter, intracellular transport**
1	234	CG6180 (*D. melanogaster*)	$2e^{-40}$	phosphatidylethanolamine binding
1	209	heat shock protein 70Cb (*D. melanogaster*)	$2e^{-64}$	chaperone binding
1	207	ecdysteroid-regulated E74 (*A. mellifera*)	$2e^{-78}$	specific RNA polymerase II transcription factor activity,

				regulation of biological process, post-embryonic development, cell differentiation, regulation of developmental process, larval or pupal development, transcription, programmed cell death
1	203	muscle-specific protein 300 (*D. melanogaster*)	$3e^{-07}$	actin binding, female germline ring canal stabilization, skeletal muscle tissue development, establishment of nucleus localization, cell migration, cytoplasmic transport, nurse cell to oocyte, actin filament organization.
1	202	sex lethal (*A. mellifera*)	$2e^{-46}$	Sex determination, translation repressor activity
1	201	CG33521 (*D. melanogaster*)	$5e^{-85}$	zinc ion binding
1	**143**	**absent, small, or homeotic discs 2 (*D. melanogaster*)**	**$9e^{-50}$**	**zinc ion binding, protein binding, imaginal disc-derived wing vein specification, chromatin-mediated maintenance of transcription, imaginal disc-derived wing morphogenesis, regulation of biological process, post-embryonic development, regulation of gene expression, epigenetic, larval or pupal development, organ development**
1	186	CG10979 (*D. melanogaster*)	$2e^{-19}$	zinc ion binding, nucleic acid binding
1	185	CG7207 (*D. melanogaster*)	$2e^{-64}$	protein serine/threonine kinase activity
1	182	similar to DumPY (*A. mellifera*): shorter than wild-type family member (*A. mellifera*)	$2e^{-33}$	serine-type endopeptidase inhibitor activity
1	130	beta amyloid protein precursor-like (*D. melanogaster*)	$4e^{-29}$	protein binding, neuron projection morphogenesis, response to axon injury, peripheral nervous system development, synapse organization, cell differentiation
3	203	hypothetical protein LOC413912 (*A. mellifera*)	$5e^{-59}$	
1	267	isotig13329 (*A. mellifera*)	$1e^{-10}$	
1	266	CG15673 (*D. melanogaster*)	$7e^{-26}$	
1	246	hypothetical protein LOC727105 (*A. mellifera*)	$3e^{-30}$	
1	193	isotig05462 (*A. mellifera*)	$4e^{-10}$	
1	188	CG15040 (*D. melanogaster*)	$7e^{-39}$	
1	97	isotig02495 (*A. mellifera*)	$8e^{-19}$	
51	**277**	**No-match**		
22	**238**	**No-match**		
12	271	No-match		
8	248	No-match		
5	207	No-match		

3	205	No-match		
2	188	No-match		
1	353	No-match		
1	228	No-match		
1	205	No-match		
1	**143**	**No-match**		
1	100	No-match		

Table IIIS - Supplementary Material

Table IIIS- RDA adapter sequences used for RDA library generation and RT-qPCR primers for expression analysis of genes identified as differentially expressed. The gene-specific primers are with their respective target genes, the length of the amplified fragments and the corresponding primer pair sequences

Primer name	Length (pb)	Sequence
RDA Adapters		R12: 5′-GATCTGCGGTGA-3′ R24: 5′-AGCACTCTCCAGCCTCTCACCGCA-3′ J12: 5′-GATCTGTTCATG-3′ J24: 5′-ACCGACGTCGACTATCCATGAACA-3′ N12: 5′-GATCTTCCCTCG-3′ N24: 5′-AGGCAACTGTGCTATCCGAGGGAA-3′
ribosomal protein 49	150	rp49R : 5'-TTGAGCACGTTCAACAATGG-3' rp49F : 5'-CGTCATATGTTGCCAACTGGT-3'
actin	156	actR: 5'-GGGTATGGAAGCCTGCGGTATC-3' actF: 5'-CGGGTGGTGCGATAATCTTG-3′
myosin regulatory light chain 2	133	Tn1F: 5'-ACATTCCGATAAGGCTTTGC-3' Tn1R: 5'-GATGGTGAAAGATTGAGGCA-3'
actin 88E	167	Tn2F: 5'-CGTGACATCAAGGAGAAACT-3' Tn2R: 5'-GGATGGTTGGAACAGGGCT-3'
40S ribosomal protein SA	146	Tn3F: 5'-CTGCCGATTGCGTTGTAGT-3' Tn3R: 5'-GGTTGCCCCTGAAAATTG-3'
troponin T	140	Tn4F: 5'-CGAAACATCCTACGAACCAT-3' Tn4R: 5'-TCCCACGACCACTCCGAT-3'
serine protease inhibitor 5	111	Tn5F: 5'-AAGATGTCTCAGATAGAATCGC-3' Tn5R: 5'-TAAGGAGTTCTCGGTAGTGC-3'
ecdysteroid-regulated gene E74	166	Tn7F: 5'-CGAATCTCTTACTGAAAGCCA-3' Tn7R: 5'-TGATGAGGATGATAGGAGGG-3'
rieske iron-sulfur protein	96	Tn10F: 5'-ATGCTGGTGATTTTGGTGGT-3' Tn10R: 5'-CTAAATTTAGAGGAGCTGGTC-3'
T2n1	148	T2n1F: 5'-CCAGACAGAACGACGCCG-3' T2n1R: 5'-TACCATTGCCGAAAGAGTCC-3'
CG32464-PK	135	T2n2F: 5'-AAGAGTGTGTAACGAAAAAGAG-3' T2n2R: 5'-CGAAAGGAAAGAAAGTGTTGTT-3'
T2n3	134	T2n3F: 5'-CGTGTTGTCGCAGAAGCAG-3' T2n3R: 5'-CAGTCATCGTCGCCGTCTT-3'
CG13377-PA	156	T2n4F: 5'-CTCATCGGTGGCATTCCTC-3' T2n4R: 5'-GTCTTACGCTCAAACACTCTG-3'

immunoglobulin like-fibronectin type III	109	T2n5F: 5’-CTTGGTCCGATGCTGGTATG-3’ T2n5R: 5’-GATTCTCCGATGCTGTTTAC-3’
CG8785-PA	197	T2n6F: 5’-CGACTGGAAGCCTGGAATC-3’ T2n6R: 5’-ACCGAAATCTGAGTTGCTGTT-3’
Calcium-transporting ATPase	169	T2n7F: 5’-GAATGAGCAAAATGACGAAGG-3’ T2n7R: 5’-CCAGCCGAGGAGGGAAAG-3’
T2n8	167	T2n8F: 5’-CCTCGTTTCGGTTCATGGG-3’ T2n8R: 5’-GGTTTCGCCACAATAGGAGA-3’
Absent, small, or homeotic discs 2	113	T2n9F: 5’-GACAGGCTAGCAGTAACTGG-3’ T2n9R: 5’-GATCCTTCTGGCATTTCTTCA-3’
CG8026-PA	202	T2n10F: 5’-TCCGCTTCTGCCCGTGCT-3’ T2n10R: 5’-GACTCCTTCACTCCTAACAAT-3’

CAPÍTULO 3

Characterization of cuticular hydrocarbons of diploid and haploid males, workers and queens of the stingless bee *Melipona quadrifasciata* (Hymenoptera: Apidae)*

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Running title: Cuticular hydrocarbons of diploid males

Key words: cuticular lipids, gas chromatography-mass spectrometry, diploid male, *Melipona quadricfasciata.*

***** Artigo escrito de acordo com as normas da revista Insectes Sociaux.

Summary

Males, queens and workers of stingless bees show differences in external morphology, behavior and roles within a colony. In addition, each individual has a cuticular chemical signature responsible for mutual communication that is essential for maintaining the integrity of the colony. In this paper we characterize the cuticular hydrocarbon composition of newly emerged diploid and haploid males, workers and queens of *Melipona quadrifasciata* by gas chromatography-mass spectrometry (GC/MS) analysis. This is the first time that the cuticular profile of diploid males in a species of stingless bees is characterized. No compound was found exclusively in these males. The cuticular chemical profiles of the haploid and diploid males were very similar to that of workers. Moreover, cuticular lipids of these males and workers were significantly different from those of queens what corroborates with the behavioral and morphological differences among these phenotypes.

Introduction

In *Melipona quadrifasciata*, as in most Hymenoptera, fertilized eggs give rise to females and unfertilized eggs originate haploid males. Fertilized eggs that show homozygosity in the sex determining locus develop into diploid males (Whiting, 1939). Especially inbreeding leads to an increasing of diploid male production supported by matched mating. Sib-mating increases the chance of such matched matings and, hence, the production of diploid males in a colony (van Wilgenburg et al., 2006). Homozygosity at the sex locus caused by inbreeding leads to 50% of fertilized eggs developing into diploid males (Crozier, 1971; Woyke, 1986). Their production is disadvantageous for the colony, because it decreases the potential worker production (Ratnieks, 1990; Roubik et al., 1996; Zayed et al., 2003; Herzner et al., 2006; Armitage et al., 2010).

Alves et al. (2011) showed that populations of *Melipona scutellaris* artificially imposed a genetic bottleneck, successfully survive for over 10 years. This fact demonstrates that breeding from a small stock of colonies, producing diploid males, may have less severe consequences than previously suspected. However, these colonies were occasionally provided with additional food and supplemented with brood from stronger colonies, may have contributed to the survival of the population.

In *Apis mellifera*, species with progressive provisioning in which larvae are fed during their development, diploid male larvae are detected and removed by worker cannibalism right after hatching (Woyke, 1963). This behavior suggests the existence of a “cannibalism substance”, produced by the diploid male larvae, which is responsible for its elimination by workers (Woyke 1967, 1980; Dietz and Lovins, 1975). In contrast, stingless bees’ cells are mass provisioned and each larva is provided with the total amount of food required for adult development before the cell is sealed (Michener, 1974). This prevents that adult workers detect and eliminate diploid male larvae at an early stage. These males can develop into adults as it was already recorded for *Bombus atratus* (Garófalo, 1973; Plowright and Pallet, 1979), *B. terrestris* (Duchateau and Marrien, 1995), *Lasioglossum zephyrum* (Kukuk and May, 1990) and the stingless bees *Trigona quadrangula*

(Tarelho, 1973), *M. quadrifasciata* (Camargo, 1982), *M. compressipes* (Kerr, 1987), *Scaptotrigona postica* (Paxton et al., 2003), *Trigona carbonaria* (Green and Oldroyd, 2003) and *Lestrimelitta* sp. (Tavares et al., 2010).

Diploid males of *M. quadrifasciata* have high viability during the development stage, but the workers replace the queen which produces these males in order to avoid the death of the colony (Camargo, 1979; Ratnieks, 1990). These males are morphologically similar to haploid males and it is difficult to distinguish them by eye within a colony. However, Camargo (1984) observed differences in the counts of spermatozoa these males: diploid males have lower spermatozoa counts that migrate from the testes to the seminal vesicles later than haploid males. Corroborating the least amounts of spermatozoa in the diploid males, Tavares et al. (2003) observed that they have shorter testes and a wider thorax than haploid males.

Cuticular hydrocarbons play an important role in insect communication, because comprise the mostly volatile substances present in the epicuticle of insects. Besides their primary role as a barrier against the loss of water (Wigglesworth, 1964), several other functions have been assigned to insect hydrocarbons since their discovery in the 1960s and early 1970s. They are related to chemical communication, nestmate recognition, species and gender recognition and task-specificity (Jackson and Baker, 1970; Howard and Blomquist, 1982; Lockey, 1988; Nelson and Blomquist, 1995; Gibbs, 2002; Howard and Blomquist, 2005; Provost et al., 2008; Blomquist and Bagnères, 2010).

Until present no comparative study has been performed to characterize the chemical profile of cuticular hydrocarbons of workers, queens, haploid males, and particularly diploid males of *M. quadrifasciata*. The aim of this paper was characterize for the first time the cuticular profile of these bees and identify chemical compounds produced by diploid males that can potentially act as suicidal signals to induce behavior aggressive of workers and thus ensure the survival of the colony.

Materials and methods

Bees

Workers, queens and males were collected from *Melipona quadrifasciata* colonies kept at the meliponary of the Federal University of Viçosa (Viçosa – MG, Brazil). Diploid males were obtained from nine inbred colonies of *M. quadrifasciata* originated from a brother-sister mating. The ploidy of males obtained by inbreeding was determined by cytogenetic analysis according to Imai et al. (1988). Queens, haploid males and workers were collected from combs taken from six, nine and twelve non-inbred colonies, respectively. Entire brood frames with late pupal stages were removed and maintained in an incubator at 28^{o}C. Pairs of wings of 10 newly emerged individuals (4-6 hours) of each phenotype were carefully removed, stored in a same glass vials and kept at 4^{o}C until chemical analysis.

As in many other studies (Abdalla et al., 2003; Kerr et al., 2004; Jungnickel et al., 2004; Francisco et al., 2008), we used the wings of the bees to estimate the relative chemical composition of the cuticle of *M. quadrifasciata*. As the wings exhibit the same surface hydrocarbon as the rest of the body and are less contaminated with resins or pollen, they are appropriate for the analysis of cuticular hydrocarbons. To obtain sufficient quantities of hydrocarbons, we used a pool of 10 pairs of wings of individuals from a single colony, while most studies utilized the wings of individual bees using the Keele solid-sampling method (Morgan and Wadhams, 1972; Morgan, 1990).

Cuticular hydrocarbon extractions and GC-MS analyses

The substances present in the lipid layer of the wings of females and males of *M. quadrifasciata* were extracted for 1 min in 1 ml of hexane. Thereafter, the wings were removed from the glass vial, the solvent was allowed to evaporate under N_2 flow and the cuticular substances were suspended in 50 µl of hexane. We then injected 1 µl of this extract in a combined gas chromatography-mass spectrometry (GC-MS; Shimadzu, model QP2010). A DB-5MS capillary column, with helium as carrier gas at a

flow rate of 1 ml/min was used. The oven temperature increased from 150°C to 280°C at 3°C/min and was then held at 280°C for 15 min. The mass spectrometer operated in the electron ionization mode at 70 eV with an ion source temperature of 250°C. Analyses were performed in splitless mode and individual mass spectra were compared with Wiley Library data (GCMSsolutions for Windows, Shimadzu Corporation) and with a standard solution of different synthetic hydrocarbons.

Statistical analysis

For statistical analysis, the area of one specific peak was proportional to the concentration of the specific hydrocarbon represented by this peak in the injected samples. The peak areas of identified substances were compared by multivariate analysis. Prior to statistical analysis (Statistica 7.0 - Statsoft, Inc.), the area of each peak was corrected using Reyment's formula: $Z = \ln[Ap/g(Ap)]$, where Ap is the area of the peak, and g(Ap) is the geometric mean peak area (Aitchison, 1986).

Principal component analysis (PCA) was used to reduce the number of variables to be compared. Only peaks that had the highest factorial weight on the first two factors were selected for further statistical analysis. Subsequently, stepwise discriminant analysis was carried out to verify the differences in the cuticular hydrocarbon composition among the samples of each phenotype analyzed.

A comparison of 24 cuticular hydrocarbons shared by haploid and diploid males was made using the non-parametric Mann-Whitney's U-test to verify whether there were differences in the chemical profile of these males. The same test was used to check for differences in the composition of cuticular hydrocarbons of males, workers and queens in relation to the 21 compounds shared by all phenotypes. The statistical analyses were performed using the software Statistica 7.0 (Statsoft - Inc.).

Results

The GC/MS analysis of cuticular extracts of queens, workers, haploid and diploid males of *M. quadrifasciata* revealed a total of 42 compounds with chain lengths between C_{21} and C_{31} (Table 1). These cuticular hydrocarbons were mostly linear alkenes (50%), but linear alkanes (23.8%) and methyl-branched alkanes (23.8%) also occurred, as well as lower amounts of alkadienes (2.4%). Figure 1 shows a comparison of the cuticular hydrocarbon profiles from wings of the four different phenotypes analyzed. Most of the cuticular hydrocarbon components occurred in all four phenotypes, but showed a different pattern. The most abundant compound on the cuticular surface of all phenotypes was pentacosane followed by tricosane, pentacosene-2, heptacosene-2, heptacosane, and nonacosene-2 (Peaks 5, 2, 4, 7, 9 and 16, respectively as shown in Figure 1) with different percentages of occurrence in queens, workers, diploid and haploid males.

The general pattern for cuticular extracts of diploid males corresponded to that of haploid males, workers and queens. No substance occurred exclusively in the cuticle of the diploid males. Some compounds, although in lower quantities (less than 0.25%), were found only in haploid males (*ni*-Methyl-Octacosane and 5-Methyl-Octacosane), workers (Pentacosene-1, *ni*-Methyl-Pentacosane) or queens (Nonacosene-4). Nonacosene-1 was exclusively found in the queens' extracts, in concentrations of 3.84%. In addition, it should be noted that the queens' profile differed in more compounds from the other phenotypes' profiles (Table 1).

Diploid and haploid males shared almost all cuticular substances, except tetracosene-1 which was found only in diploid males and *ni*-methyl-octacosane and 5-Methyl-Octacosane present only in haploid males (Table 1). No significant differences were found between haploid and diploid males in the relative abundance of these compounds (Mann-Whitney's U-test, $P \geq 0.05$) when 24 compounds shared by them were analyzed.

Although six eigenvalues of correlation matrix were higher than 1 explaining 87.19% of the total variation, two factors explained the variation between the predicted groups. The first and second principal components

described 31.5% and 20.83% of the total variance, respectively. Univariate analysis did not revealed any differences in the chemical profiles of haploid and diploid males, as well as between diploid males and workers (Mann-Whitney's U-test, $P \geq 0.05$). However, the comparison of diploid males and queens revealed a significant difference of the cuticular hydrocarbons profiles. Tricosane, 5-methyl-pentacosane, heptacosene-3 and octacosene-3 were the most important compounds that contributed for the discrimination of these two phenotypes (Mann-Whitney's U-test, $P \leq 0.05$). These same compounds with addition of hexacosene-2 were similarly able to distinguish haploid males from queens (Mann-Whitney's U-test, $P \leq 0.05$). Only nonacosane was responsible for the differentiation of haploid males and workers (Mann-Whitney's U-test, $P \leq 0.05$). Finally, workers were distinguished from queens by the presence of hexacosene-1, heptacosene-3 and nonacosene-2 (Mann-Whitney's U-test, $P \leq 0.05$).

A discriminant analysis based on the six principal components, revealed that the cuticular hydrocarbons profiles of queens and others phenotypes significantly differed (Wilk's $\lambda = 0.03568$; $F_{42,57} = 2.8237$; $p \leq 0.001$). The most important hydrocarbons responsible for the significant separation of queens from other groups were tricosane, pentacosene-2 and *ni*-methyl-heptacosane. The classification based on the discriminant analysis separated 100% of all samples correctly as queen and diploid males, 88.8% as haploid males and 83.3% as workers. The canonical analysis demonstrated that the newly emerged queens group was clearly segregated from the group formed by workers and males (Figure 2). This result was supported by the chemical distance (Table 2). Furthermore, diploid males were more closely related to workers than to haploid males, although this result was not statistically significant.

Discussion

The cuticle of newly emerged females and males of *M. quadrifasciata* had similar chemical profiles with an abundance of unsaturated than saturated hydrocarbons (Table 1), which is consistent with the data obtained

in *Frieseomelitta varia* (Nunes et al., 2009). In contrast, in newly emerged females and haploid males of *M. bicolor* (Abdalla et al., 2003) and *M. scutellaris* (Kerr et al., 2004), saturated hydrocarbons were the most prevalent compounds in newly emerged individuals. Such differences in the cuticular profiles of bees confirm that the chemical profile of an insect cuticule is a helpful tool for the detection of species-specific cues.

Similarly with previous studies of cuticular hydrocarbons of other stingless bee species (Abdalla et al., 2003; Kerr et al., 2004; Jungnickel et al., 2004; Pianaro et al., 2007; Francisco et al., 2008; Nunes et al., 2009, Ferreira-Caliman et al., 2010) the most common compounds among the cuticular hydrocarbons of newly emerged individuals of *M. quadrifasciata* were tricosane, pentacosane, heptacosene, heptacosane and nonacosene, although the relative ratios of these compounds differ among these species. According to Wigglesworth (1970), these substances are probably structural hydrocarbons, since they were found in the cuticle of queens, workers and males. Analysis of the cuticular chemical profile of adults may reveal whether such composition has no changes throughout adulthood indicating that they are indeed structural.

For the human eye it is almost impossible to distinguish haploid and diploid males within the colony of *M. quadrifasciata* and the chemical profile confirmed the similarities between them. Except for tetracosene-1 which was found only in diploid males and *ni*-methyl-octacosane and 5-Methyl-Octacosane present only in haploid males, all other cuticular substances are shared for these males (Table 1). Hermann et al. (2005) observed that the general pattern obtained for third-instar larvae extracts of the diploid males of *Apis mellifera* corresponded to those of haploid males.

Our study showed that, although not statistically significant, the cuticular profiles of wings of haploid and diploid males were more similar to workers than to queens. This result corroborates the male/worker similarities in the behavior of the stingless bee. Males of Meliponinae, Bombinae and also in some Halictidae sporadically perform activities that in *Apis* are reserved only to the workers such as nectar dehydration, wax manipulation, trophallaxis, feeding the queen and foraging (Silva, 1977; Cortopassi-

Laurino, 1979; Engels and Engels, 1988; Kerr, 1997; Nogueira-Neto, 1997; Veen et al., 1997; Velthuis et al., 2005).

According to the chemical distance, diploid males were more closely related to workers than to haploid males (Table 2). This result can be explained by diploid individuals presenting the same ploidy and similar levels of gene expression which can reflect in the production of similar enzymes that regulate the biosynthetic pathways of insect hydrocarbons (Kerr et al., 2004; Blomquist and Bagnères, 2010).

Otherwise, cuticular lipids of males and workers were significantly different from those of queens (Table 2). These differences in cuticular chemical signature of males, workers and queens corroborate the behavioral and morphological distinctions among these phenotypes. A similar characterization was found in *M. bicolor* (Abdalla et al., 2003), *M. scutellaris* (Kerr et al., 2004) and *F. varia* (Nunes et al., 2009). In all of these studies the cuticle of queens was different from that of haploid males and workers. Based only on morphological characteristics such as length of posterior wing, width of mesoscutum, total length of head and ocellorbital distance, it was observed that stingless bee workers were more similar to males than to queens (*M. compressipes* and *M. marginata* – Bonnetti and Kerr, 1985; *M. quadrifasciata* – Campos, 1978; and *M. scutellaris* – Almeida, 1985).

In the present study, the cuticle profile of diploid male of a stingless bee species was analyzed for the first time. No compound was found exclusively in these newly emerged males. A similar result was obtained by Santomauro et al. (2004) who studied the cuticular hydrocarbons composition of diploid male larvae in the honeybee. They observed that a quantitative shift in the pattern of tricosane, pentacosane, heptacosane, nonacosane and terpenoid was enough for the nurse bees recognize and kill diploid male larvae.

Unlike the progressive provisioning system of honeybees where brood is reared in open cells (Woyke, 1963), brood cells in stingless bees *M. quadrifasciata* are mass-provisioned upon which the queen lays an egg during the provisioning and oviposition process (POP) and thereafter the cell is sealed (Sakagami and Zucchi, 1974). This prevents that diploid male larvae are eliminated by adult worker at the initial stage. Thus, diploid male

larvae can reach adulthood (Camargo, 1982). Nevertheless, the workers detected them and replace the queen who is producing these males (Ratnieks, 1990).

Although no compounds have been found specifically in the newly emerged diploid males, workers of *M. quadrifasciata* recognize these males and they deal in a different way from that of *A. mellifera*. Therefore further research is needed to check whether the cuticular composition of females and males, especially of diploid males, changes throughout the adulthood. This allows the identification of specific compounds or quantitative patterns that can act as suicidal signals to help the workers to be recognize these males and thus ensure the survival of the colony.

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Table 1. Relative concentrations ($\overline{X}$ ± SD) of cuticular hydrocarbons of queens, workers, haploid (n) and diploid males (2n) of *M. quadrifasciata*

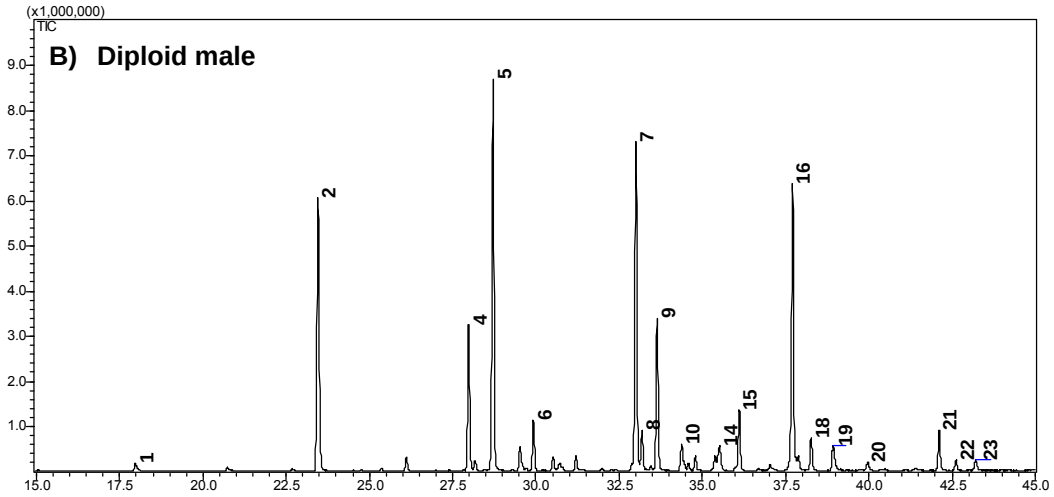
Peaks	RT (min)	Compounds	Queen	Worker	Male n	Male 2n
1	17.98	Heneicosane (C_{21})	-	0,69 ± 0,49	0,42 ± 0,33	0,84 ± 0,74
2	20.75	Docosane (C_{22})	-	0,21 ± 0,07	0,59 ± 1,14	0,20 ± 0,08
3	22.68	Tricosene	-	0,19 ± 0,12	0,11 ± 0,01	0,14 ± 0,07
4	23.47	Tricosane (C_{23})	5,35 ± 5,39	10,66 ± 5,60	12,01 ± 4,56	14,01 ± 4,18
5	24.77	Tetracosene-1	-	3,01 ± 4,97	*trace*	0,11 ± 0,01
6	25.37	Tetracosene-2	-	0,23 ± 0,13	0,11 ± 0,01	0,14 ± 0,03
7	26.12	Tetracosane (C_{24})	0,59 ± 0,46	0,55 ± 0,18	0,62 ± 0,16	0,67 ± 0,18
8	27.85	Pentacosene-1	-	0,15 ± 0,05	*trace*	-
9	27.99	Pentacosene-2	3,15 ± 1,18	5,66 ± 3,41	7,35 ± 5,08	5,68 ± 2,81
10	28.19	Pentacosene-3	0,2 ± 0,05	0,48 ± 0,16	0,52 ± 0,25	0,41 ± 0,19
11	28.72	Pentacosane (C_{25})	19,39 ± 10,15	22,02 ± 5,17	20,06 ± 6,91	21,42 ± 4,33
12	29.54	11, 13-Methyl-Pentacosane	0,43 ± 0,07	0,69 ± 0,44	0,80 ± 0,39	0,77 ± 0,33
13	29.72	*ni*-Methyl-Pentacosane	-	0,12 ± 0,02	*trace*	*trace*
14	29.94	5-Methyl-Pentacosane	1,21 ± 0,34	1,75 ± 0,75	2,07 ± 0,54	2,25 ± 0,72
15	30.53	Hexacosene-1	0,67 ± 0,16	0,51 ± 0,17	0,53 ± 0,17	0,56 ±0,09
16	30.72	Hexacosene-2	0,39 + 0,56	0,28 ± 0,13	0,36 ± 0,11	0,36 ± 0,13
17	30.82	ZZ-Hexacosene	Trace	0,17 ± 0,06	0,13 ± 0,01	0,15 ± 0,02
18	31.22	Hexacosane (C_{26})	0,96 ± 0,47	0,86 ± 0,35	0,64 ± 0,21	0,86 ± 0,23
19	32.88	Heptacosene-1	0,76 ± 0,48	0,26 ± 0,09	0,24 ± 0,05	0,21 ±0,07
20	33.01	Heptacosene-2	16,16 ± 6,64	12,65 ± 4,19	13,12 ± 3,85	12,66 ± 3,11
21	33.20	Heptacosene-3	0,91 ± 0,29	1,42 ± 0,47	1,96 ± 0,88	1,40 ± 0,44
22	33.47	Heptacosene	0,20 ± 0,03	0,16 ± 0,02	0,17 ± 0,05	0,16 ± 0,01
23	33.65	Heptacosane (C_{27})	13,14 ± 2,19	15,96 ± 8,85	10,62 ± 7,05	13,69 ± 6,12
24	34.40	7-Methyl-Heptacosane	1,17 ± 0,47	0,81 ± 0,32	1,05 ± 0,36	0,78 ± 0,29
25	34.47	*ni*-Methyl-Heptacosane	0,18 ± 0,03	0,13 ± 0,04	2,19 ± 6,09	0,14 ± 0,02
26	34.80	5-Methyl-Heptacosane	0,68 ± 0,22	0,72 ± 0,22	0,68 ± 0,21	0,91 ± 0,28
27	35.39	Octacosene-1	0,76 ± 0,10	0,49 ± 0,14	0,55 ± 0,19	0,48 ± 0,19
28	35.53	Octacosene-2	1,00 ± 0,41	1,18 ± 0,40	1,48 ± 0,56	1,47 ± 0,44
29	36.01	Octacosene-3	0,73 ± 0,76	0,29 ± 0,09	0,28 ± 0,19	0,27 ± 0,10
30	36.11	Octacosane (C_{28})	4,86 ± 4,35	1,95 ± 1,89	3,54 ± 2,58	2,12 ± 2,52
31	36.69	*ni*-Methyl-Octacosane	*trace*	*trace*	0,16 ± 0,04	*trace*

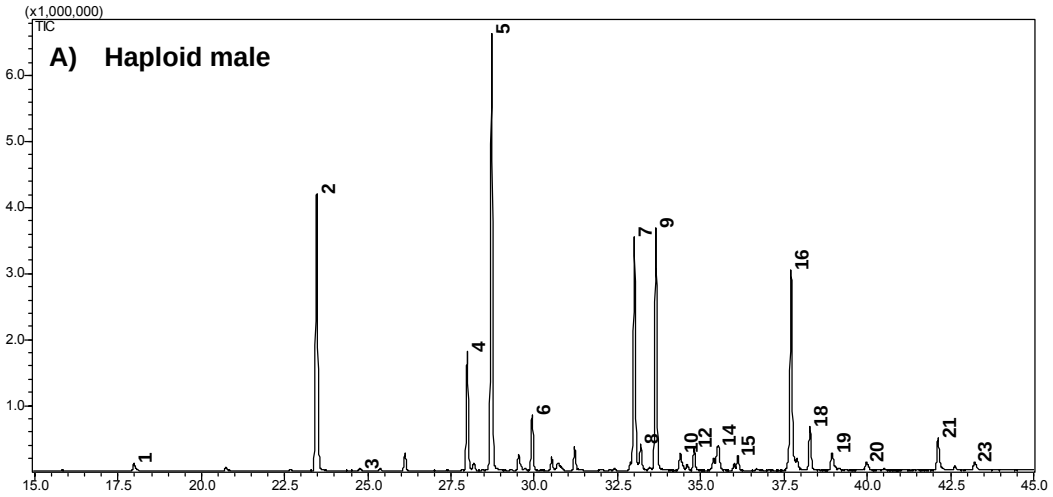
32	37.04	5-Methyl-Octacosane	-	*trace*	0,20 ± 0,01	-
33	37.51	Nonacosene-1	3,84 ± 2,51	-	*trace*	-
34	37.72	Nonacosene-2	17,30 ± 7,85	11,24 ± 3,45	12,04 ± 3,32	10,33 ± 2,78
35	37.88	Nonacosene-3	2,49 ± 1,97	0,68 ± 0,26	0,74 ± 0,24	0,48 ± 0,24
36	37.98	Nonacosene-4	0,22 ± 0,14	*trace*	*trace*	*trace*
37	38.28	Nonacosane (C_{29})	2,76 ± 1,22	4,05 ± 3,70	1,94 ± 2,01	3,14 ± 1,94
38	38.94	*ni*-Methyl-Nonacosane	1,11 ± 0,34	0,90 ± 0,38	1,06 ± 0,36	0,91 ± 0,30
39	39.98	Triacontene	0,54 ± 0,22	0,36 ± 0,09	0,41 ± 0,11	0,41 ± 0,08
40	42.13	Hentriacontene	2,87 ± 2,61	1,50 ± 0,43	1,36 ± 0,38	1,65 ± 0,35
41	42.63	Hentriacontane (C_{31})	0,60 ± 0,30	0,93 ± 1,29	0,59 ± 0,40	0,66 ± 0,55
42	43.22	*ni*-Methyl-Hentriacontane	0,46 ± 0,17	0,39 ± 0,09	0,41 ± 0,15	0,32 ± 0,15

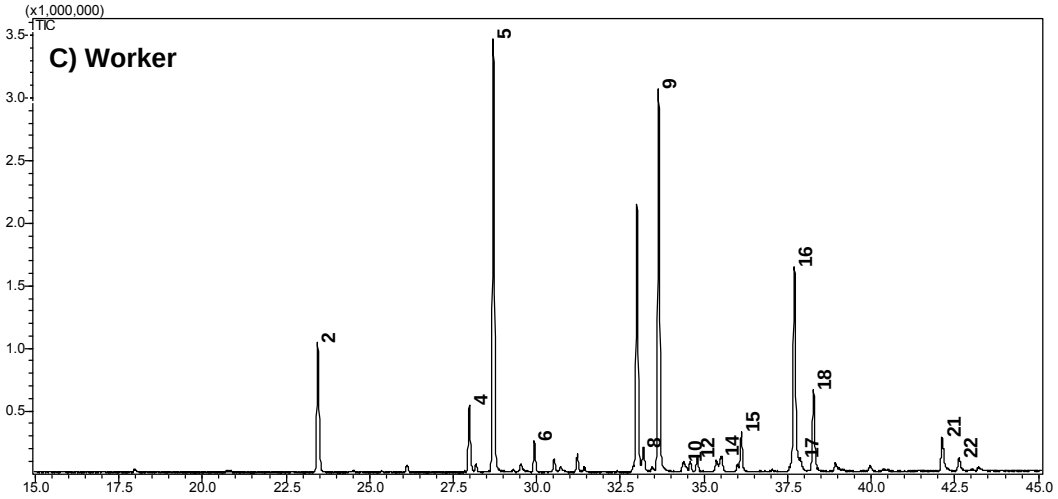
RT: Retention time; *ni*: position of methyl groups not identified; -: Compounds absent; *trace*: compounds present in only 2 to 3 colonies analyzed.

Table 2. Discriminant analyses for queens, workers, diploid (2n) and haploid (n) males of *M. quadrifasciata* (Wilks' λ= 0.03568; $F_{42,57}$= 2.8237; p<0.001)

Groups	Mahalanobis distance	F-values	p-Level
Male 2n x Male n	8,89	1,69	0.1400
Male 2n x Worker	7,19	1,57	0.1779
Male 2n x Queen	39,32	6,00	0.0002
Male n x Worker	6,96	1,52	0.1953
Male n x Queen	37,40	5,71	0.0003
Worker x Queen	30,74	5,21	0.0005


(x1,000,000)
TIC
B) Diploid male


(x1,000,000)
TIC
A) Haploid male


(x1,000,000)
TIC
C) Worker

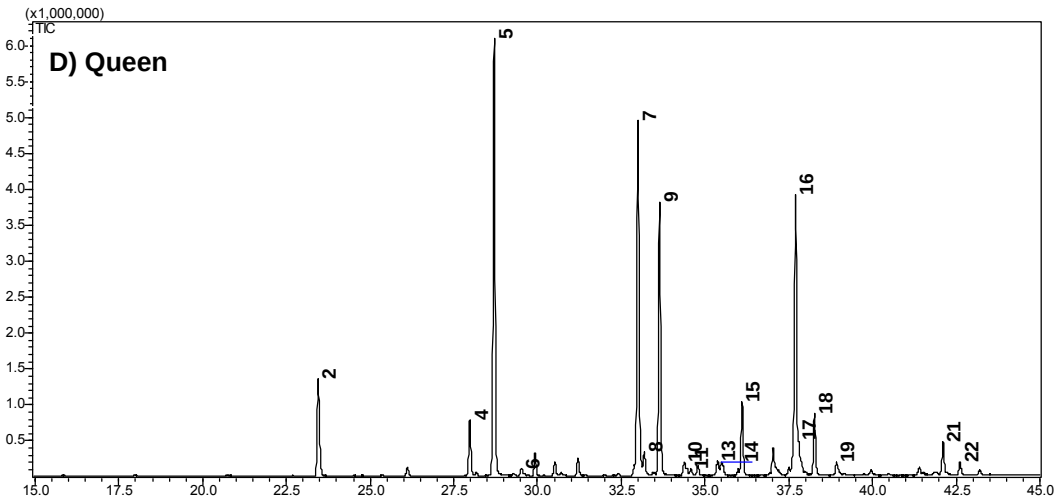

(x1,000,000)
TIC
D) Queen

Figure 1. Comparative gas chromatograms of a representative individual of diploid male (A), haploid male (B), worker (C) and queen (D) of *M. quadrifasciata*. Main peaks were: (1) heneicosane; (2) tricosane; (3) tetracosene-1; (4) pentacosene-2; (5) pentacosane; (6) 5-methyl-pentacosane; (7) heptacosene-2; (8) heptacosene-3; (9) heptacosane; (10) 7-methyl-heptacosane; (11) *ni*-methyl- heptacosane; (12) 5-methyl-heptacosene; (13) octacosene-1; (14) octacosene-2; (15) octacosane; (16) nonacosene-2; (17) nonacosene-3; (18) nonacosane; (19) *ni*-methyl-nonacosane; (20) triacontene; (21) hentriacontene; (22) hentriacontane; (23) *ni*-methyl-hentriacontane.

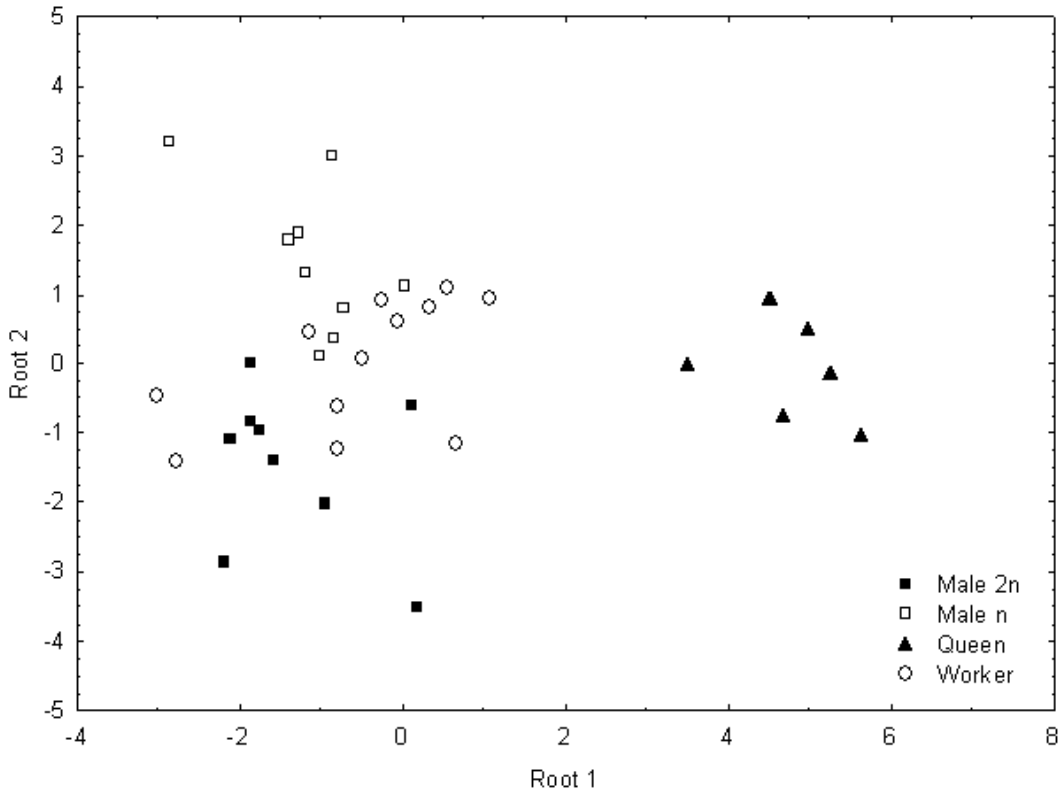


Figure 2. Plot of the first two functions of the discriminant analysis of cuticular hydrocarbons profiles of queens (N= 6), diploid (2n) and haploid (n) males (both N= 9 colonies) and workers (N= 12) of *M. quadrifasciata*.

CONCLUSÕES GERAIS

- Machos e fêmeas de *M. quadrifasciata* apresentaram diferenças nos níveis de expressão gênica.

- Principal diferença na abundância dos transcritos foi observada entre machos diplóides e rainhas.

- Foi verificada uma ausência de transcritos relacionados com processos reprodutivos nos machos diplóides o que pode explicar a sua maturação sexual tardia e menor fitness.

- Machos e fêmeas de *M. quadrifasciata* apresentaram diferentes assinaturas químicas cuticulares.

- O perfil dos hidrocarbonetos cuticulares de machos haplóides e diplóides foi mais similar aos de operárias do que ao de rainhas, corroborando as semelhanças morfológicas e comportamentais entre machos e operárias.

- Nenhum composto foi encontrado exclusivamente na cutícula dos machos diplóides recém-emergidos.