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Research Article

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

Andreia A. Borges[1], Fernanda C. Humann[2], Lucio A. Oliveira Campos[1], Mara G. Tavares[1] and Klaus Hartfelder[2]

[1]*Departamento de Biologia Geral, Universidade Federal de Viçosa, Viçosa, MG, Brazil.*
[2]*Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil.*

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. Yet, diploid males are of academic interest as they can 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* were increased during the first five days of adult life, while those for *diacylglycerol kinase* and the transcriptional co-repressor *groucho* diminished. Two general trends were apparent, (i) gene expression patterns in diploid males were overall more similar to haploid ones and workers than to queens, and (ii) in queens and workers, more genes were up-regulated after emergence until day five, 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 longer in females than in males.

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

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Introduction

Sex and caste determination in stingless bees are contentiously 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).

Leaving aside its initial triggers, differentiation of the caste phenotypes in 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 and ecdysteroid titers (Rachinsky *et al.*, 1990), as well as insulin signaling (Wheeler *et al.*, 2006; Azevedo and Hartfelder, 2008).

While the developmental processes and roles of the queen and worker castes in division of labor within colonies of highly eusocial bees are fairly well understood, only 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 develop from fertilized eggs. The mechanism underlying sex determination in haplo-diploid 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 is a determinant for the male sex. 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 as a result of latent inbreeding and consequent allelic matches at the sex locus

Send correspondence to Klaus Hartfelder. Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Avenida Bandeirantes 3900, 14049-900 Ribeirão Preto, SP, Brazil. E-mail: klaus@fmrp.usp.br.

(van Wilgenburg *et al.*, 2006). The *csd* locus has been mapped and sequenced in the honey bee (Beye *et al.*, 2003) and was 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 signal male and female Doublesex (Dsx) isoforms produced through alternative splicing (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 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 stage. Nursing workers perceive these through a "cannibalization signal" (Woyke, 1967), which is thought to represent 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 an 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 a window of 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, 1963). Diploid males of *Melipona quadrifasciata* were first successfully produced by Camargo (1974, 1979) through a brother-sister mating protocol, and this has aided research on their life history (Camargo, 1982).

Behavioral, morphological, and cuticular hydrocarbon 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 so because they cannot easily be distinguished from the normal haploid ones; this only being 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 among haploid and diploid males on the one hand, and the differences in life span and fertility on the other made us ask as to whether such discrepancies might correlate with differences in gene expression patterns. In the present study we investigated transcript levels of ten genes previously identified as differentially expressed in the female castes of the stingless bee *Melipona quadrifasciata* (Judice *et al.*, 2004, 2006), comparing their relative expression levels 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, viz. on day 0, shortly after they 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 covers the period of adult sexual maturation, including the migration of spermatozoa 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, this being as a sharp peak in the ecdysteroid hemolyph titer that occurs in conjunction with a broader peak in the juvenile hormone titer (Santana and Hartfelder, unpublished results).

Materials and Methods

Bees and total RNA extraction

Males and females of the stingless bee *Melipona quadrifasciata* were collected from colonies kept at the meliponary of the Federal University of Viçosa, Viçosa, MG, Brazil. Haploid males, workers and queens were retrieved from combs taken from strong colonies which did not show signs of inbreeding, whereas diploid males were obtained from inbred colonies set up through a brother-sister mating scheme (Camargo, 1974, 1979). Ploidy of these males was determined by cytogenetic analysis (Imai *et al.*, 1988).

Brood combs containing late pupal stages were removed from the colonies and kept in an incubator at 28 °C. Day-0 bees were collected within 4-6 h after emerging from the brood cells. To assess transcript abundance in 5-day-old adults, such newly emerged bees were kept in Petri dishes in an incubator (28 °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 °C.

For RNA extraction, two individuals of each type and age class were pooled and homogenized in 1 mL of TRIzol reagent (Invitrogen) following the manufacturer's protocol. The resultant RNA pellet was resuspended in diethyl

pyrocarbonate-treated water. A DNase treatment (0.1 U RNase-free 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 run 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. The first four encode a transmembrane transporter (TM-transporter), a permease, a ceramide kinase, and a gene of unknown function, provisionally named *Mq5.* They were chosen from a gene set revealed as differentially expressed in a custom-made microarray for *M. quadrifasciata* (Judice, Pereira and Hartfelder, unpublished).

The gene herein named *TM-transporter* corresponds to *a Melipona* RDA library EST sequence (GenBank accession number CO729459.1); it has sequence similarity with the *Drosophila* gene CG1607 which encodes a predicted transmembrane transporter. The gene named *permease* corresponds to a *Melipona* RDA library EST sequence (GenBank accession number CO578735.1); it is similar to the *Drosophila* gene CG2316 which has as Gene Ontology molecular function predictions: ATPase activity, coupled to transmembrane movement of substances, these characterizing it as a permease. The gene named *ceramide kinase* corresponds to a *Melipona* RDA library EST sequence (GenBank accession number CO578687.1); it is similar to the *Drosophila* gene CG16708, *Cerk*, which has experimentally validated ceramide kinase activity. The gene named *Mq5* is a *Melipona* RDA library sequence (GenBank accession number CO578601.1); it is an EST without significant similarity to genes of other organisms. EST clones for these four genes were re-sequenced and gene identification checked by blastx and blastn searches against GenBank sequences and the dbEST database for *Melipona 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 for ESTs encoding dunce (GenBank accession number CO729450.1), paramyosin (CO729458.1), groucho (CO729465.1), an amino acid-polyamine transporter (AAP-transporter; CO729459.1), a fatty acid synthase (FAS; CO729464.1) and diacylglycerol kinase (DGK; CO729466.1), 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.

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

Target gene	Primer	Sequence	Source
Ribosomal protein 49	rp49F	5'-CGTCATATGTTGCCAACTGGT-3'	Lourenço *et al.*, 2008
	rp49R	5'-TTGAGCACGTTCAACAATGG-3'	
Actin	ActMelF	5'-CGGGTGGTGCGATAATCTTG-3*'*	Judice *et al.*, 2004
	actMelR	5'-GGGTATGGAAGCCTGCGGTATC-3'	
Transmembrane transporter	Mq1F	5'-TTGCTATCTCGTGCGTCTTG-3'	This study
	Mq1R	5'-GCGAACATGCCGAATAAACG-3'	
Permease	Mq2F	5'-CCCGTTAGATGCGACTCAG-3'	This study
	Mq2R	5'-CATGCTTGTCCGTTTCATATTG-3'	
Ceramide kinase	Mq3F	5'-CACGTTCGTGGTGAAGAAGA-3'	This study
	Mq3R	5'-CCTCGTGTATCCAATCGTCC-3'	
Mq5	Mq5F	5'-GGATTTCAAAGTGGCTGGC-3'	This study
	Mq5R	5'-GTTAATCACGTTCAATCGCCC-3'	
Dunce	DunF	5' -AGCCGACCTGCGACTTCTC-3'	Judice *et al.*, 2006
	DunR	5' -ACATGGACATTAGCCCAATGTG-3'	
Amino acid-polyamine transporter	AmitranspF	5' -AGGGAAGATCCCGTCAAGAA-3'	Judice *et al.*, 2006
	AmitranspR	5' -GGGTCGTGTAAAATGCCATGT-3'	
Paramyosin	PmyosF	5' -ATCCGAGGGAAGATCCAGGTA-3'	Judice *et al.*, 2006
	PmyosR	5' -TGCCTCTTGTAGATGCTCATTTTC-3'	
Diacylglycerol kinase	DGKF	5' -CTTCGTATCGATGCCAGCAA-3'	Judice *et al.*, 2006
	DGKR	5' -TTTTGTTGTTCGTCAATCCGTTT-3'	
Fatty acid synthase	FASF	5' -GATCGCGGGATTGATACCTACT-3'	Judice *et al.*, 2006
	FASR	5' -TCGACGGTAACAAAAGTCAAGGA-3'	
Groucho	GrouF	5' -CGGCGGACGGTTCGA-3'	Judice *et al.*, 2006
	GrouR	5' -GATCCCACGAACGCACTGT-3'	

First strand cDNA templates were synthesized from 5 μg of DNase-treated RNA using Superscript II (Invitrogen) reverse transcriptase and oligo $(dT)_{12-18}$ primer (Invitrogen). Subsequently, optimal cDNA quantities were established for the target, as well as the 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 consisted of 7 μL of SYBR Green (Applied Biosystems), 1 μL of cDNA (diluted 1:10) and 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 give the expression ratio (fold change) for each gene at day 5 in relation to day 0.

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 down-regulation 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 diploid males (Table 3), expression levels continued to be lower for *ceramide kinase* and *DGK*, and were now also lower for *groucho* when compared to haploid males. Workers had lower expression levels for two genes (*dunce* and *paramyosin*) than diploid males. In contrast, 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 denoted in the directionality of age-related change in *TM-transporter, ceramide kinase* and p*aramyosin* expression, the first two being up and the latter down-regulated in 5-day-old queens and workers. A second

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 ≤ 0.05) are shown.

Gene	Haploid male	Worker	Queen
Transmembrane transporter		3.01 x down	1.64 x up
Permease			2.42 x up
Ceramide kinase	2.08.x down	2.86 x down	
Mq5			2.31 x up
Dunce			2.90 x up
Amino acid -polyamine transporter			2.,67 x up
Paramyosin	2.19 x up		2.16 x up
Diacylglycerol kinase	2.05 x down		
Fatty acid synthase	2.01 x up		2.38 x up
Groucho		4.34 x down	

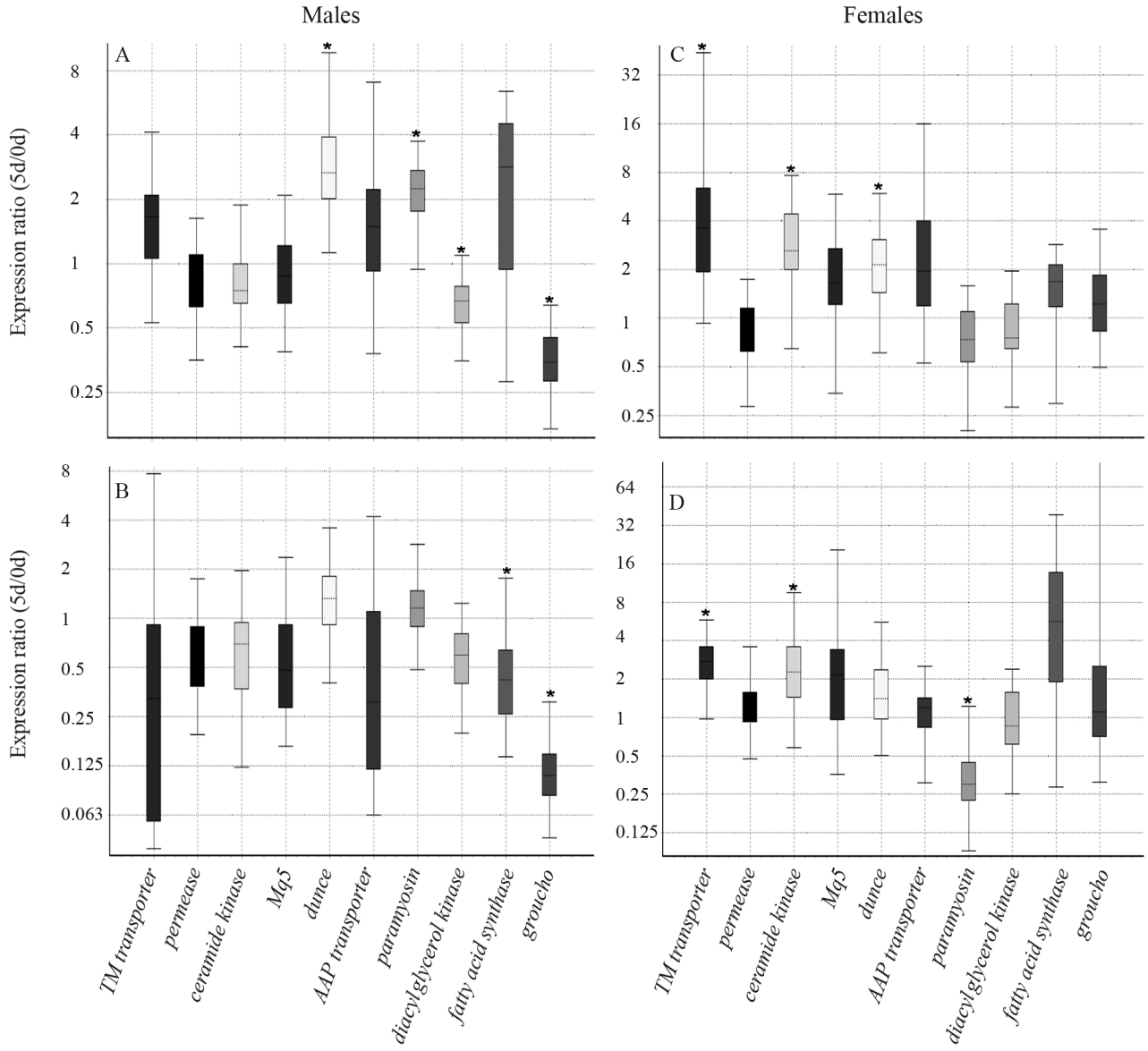

Figure 1 - Changes in gene expression levels during early adult life of *Melipona quadrifasciata* diploid males (A), haploid males (B), workers (C) and queens (D). Fold change values refer to transcript levels detected in five-day old bees in comparison to newly emerged ones (the latter set as equal 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, $p \leq 0.05$).

major conclusion was that diploid males are much more similar to haploid males and workers in the 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 life.

Before entering the discussion, a *caveat* needs to be brought up at this point. 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 rRNA as reference we followed a more stringent recommendation (Bustin *et al.*, 2009) and used two reference genes, one encoding a cytoplasmatic 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*

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. 85 x up
Permease			3.59 x up
Ceramide kinase	2.84 x down		1.91 x up
Mq5			5.65 x up
Dunce		1.95 x down	
Amino acid -polyamine transporter			
Paramyosin		1.82 x down	3.17 x down
Diacylglycerol kinase	2.37 x down		
Fatty acid synthase			5.13 x up
Groucho	1.99 x down		4.61 x up

al., 2002) we employed a stringent statistical analysis procedure specifically developed for quantitative gene expression analysis.

Discussion

Among the ten genes for which we quantified expression levels, *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 transcription was slightly up-regulated in haploid males (Figure 1), is in accordance with flight muscle maturation in preparation for mating flights of these males (van Veen *et al.*, 1997), but it was rather surprising to see this trend as well in diploid ones, as the latter 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* transcription, as it is less expressed in haploid males when compared to diploid ones. Ploidy levels may also be associated with *diacylglycerol kinase (DGK)* expression rates, as indicated through comparisons of haploid with 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* affect male sexual behavior, and *dunce* females were 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 observation in mind that *dunce* transcript levels were lower 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, Groucho Marx. 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 divergent picture in relation to females, with *groucho* transcript levels being significantly lower in newly emerged workers in comparison to diploid males (Table 2), but 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 changes in expression levels were higher than 1 for seven out of the ten genes at day five (Figure 1). In contrast eight of the ten genes were less expressed in 5-day-old haploid males, and five out of ten were so in diploid males. Possibly, this difference between the sexes may be related to life span, which is much longer 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.

Diploid males are "misfits" in terms of hymenopteran population genetics (van Wilgenburg *et al.*, 2006), and even more so in social species, where 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 no such predictions exist 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. As we could show herein, candidate genes taken from a suppression subtractive library for *M. quadrifasciata* queens and workers (Judice *et al.*, 2006) varied in their transcript levels in haploid and diploid males, which differ in ploidy but not in morphology, as well as in the female castes which differ in morphology but not in ploidy. Further investigating such gene sets may thus provide fundaments towards building gene networks underlying life history divergence in the castes and sexes of highly social insect.

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