FACULDADE DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOLOGIA

EFEITO DO PÓLEN DE EUCALIPTO GENETICAMENTE MODIFICADO EM ABELHAS *Scaptotrigona bipunctata* (MELIPONINI) e *Apis mellifera* (APINI)

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Mariana Zaniol Fernandes
Orientadora: Dra. Betina Blochtein

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SUMÁRIO

AGRADECIMENTOS

Sinto-me privilegiada por finalizar mais uma etapa de minha vida. Desta forma, gostaria de destinar um agradecimento especial à pessoas que marcaram estes últimos dois anos:

Aos meus pais, Maria do Carmo e Ricardo. Sinto-me honrada por ser filha de vocês. O suporte dado a mim diariamente, foi essencial para que eu concluísse com sucesso mais esta etapa. Poder contar com o amor e afeto de vocês é o que me faz seguir em frente.

Ao meu irmão, Ricardo, que apesar de ser o caçula, muitas vezes tomou o lugar de irmão mais velho, aconselhando-me e motivando-me a seguir adiante.

Ao meu noivo, Leonardo, agradeço pelo imenso apoio em todos os momentos em que precisei. Teu amor, carinho, dedicação e compreensão foram fundamentais ao longo deste processo.

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EPÍGRAFE

"Atrás destas páginas, mostro como vive um povo. São os membros de uma nação dinâmica e operosa, cujas origens remontam a milhões de anos de evolução lento e persistente. Se o leitor trata a Natureza a ferro e fogo, em minutos o seu machado destruidor arrasará a cidade e seus habitantes, Se, porém, o seu coração for amigo das maravilhas que nos proporcionou o Criador, preservará este pequeno reino e poderá entender melhor a Terra e a vida que a povoa. A salvaguarda dos recursos está em suas mãos."

(Nogueira-Neto, Paulo. Vida e Criação de Abelhas Indígenas Sem-Ferrão. 1997. Urna Edição Nogueirapis, 446pp.)

RESUMO

EFEITO DO PÓLEN DE EUCALIPTO GENETICAMENTE MODIFICADO EM ABELHAS *Scaptotrigona bipunctata* **(MELIPONINI) e** *Apis mellifera* **(APINI)**

As florações em massa das árvores de eucalipto são atrativas às abelhas para a coleta de recursos florais, principalmente néctar e pólen. O amplo espectro geográfico de espécies de *Eucalyptus* e a recente geração de árvores geneticamente modificadas no mundo, especialmente no Brasil e EUA, enfatiza a necessidade de informações sobre os riscos ambientais, incluindo potenciais efeitos às abelhas. Uma vez que os eventos transgênicos são expressos sob a forma de proteínas, o pólen torna-se a principal via de exposição de proteínas transgênicas às abelhas. O presente estudo objetiva avaliar os efeitos da ingestão de pólen proveniente de áreas florestais com árvores de eucalipto geneticamente modificado (GM) no desenvolvimento de *Apis mellifera* e *Scaptotrigona bipunctata.* Para tanto, avaliou-se o sincronismo no desenvolvimento de *Apis mellifera*, a morfometria de *A. mellifera* e *S. bipunctata* em estágio pupal e a massa corporal de imaturos de *S. bipunctata*. **Experimento com** *A. mellifera*: em fevereiro e março de 2011, o estudo foi conduzido em um horto experimental com eucalipto GM e em horto comercial com árvores convencionais, livre de eucalipto GM. Colmeias de *A. mellifera* foram colocadas em triplicatas no centro do horto experimental, e a 400 m, 1.000 m e 85 Km. Favos de cria foram coletados e as pupas foram avaliadas quanto ao estágio do desenvolvimento, largura da cabeça (LC) e distância intertegular (DI). **Experimento com** *S. bipunctata*: em laboratório, minicolônias de *S. bipunctata* (n=6) foram submetidas a alimentação com pólen de eucalipto GM e com pólen de eucalipto não-GM. O pólen foi obtido a partir de coletores de pólen instalados em três colmeias de *A. mellifera* instaladas no horto experimental e três colmeias no horto comercial. Foram coletadas de 10 a 15 pupas/ favo, das quais foram obtidos o massa corporal e as medidas da LC e DI. Para *A. mellifera*, verificou-se similaridade nas proporções dos distintos estágios de desenvolvimento em todas as áreas. Infere-se regularidade da postura das rainhas de *A. mellifera* no período, pois o padrão espacial-temporal da postura ocorreu conforme o esperado. Com relação as análises morfometricas, diferenças foram observadas. A média da DI das pupas localizadas a 1.000 m diferiu do valor encontrado para pupas das colmeias localizadas no horto experimental e a 85 km. Diferenças também foram observadas em pupas de colmeias localizadas a 400 m e 85 km. Consideram-se previstas as diferenças evidenciadas, pois *A. mellifera* representa populações hibridas no Brasil, com expressão fenotípica entre indivíduos. Como as diferenças verificadas não estão progressivamente relacionadas à distância da área experimental, descarta-se a hipótese de alterações ocasionadas por ingestão de pólen GM. No que refere-se às características morfometricas obtidas para *S. bipunctata*, diferenças foram observadas. No entanto, o coeficiente de variação para todas as minicolônias foi menor que 10%, indicando grupos homogêneos. Portanto tais diferenças podem ser reflexo da variação genética entre as distintas populações. As análises da massa corporal das pupas de *S. bipunctata*, indicou semelhança, as quais apresentaram em média 0,0212 g (SD+-0,0028 g) quando alimentadas com pólen de eucalipto GM e 0,0204 g (SD+-0, 0019 g) quando alimentadas com pólen de eucalipto não-GM. De acordo com o resultado, a semelhança encontrada na massa corporal das pupas alimentadas com ambos os tratamentos sugere que o efeito do pólen GM manteve-se semelhante a sua isolínea não-GM, não acarretando prejuízo às abelhas. Desta forma descarta-se a hipótese de alterações ocasionadas por consumo de pólen GM.

ABSTRACT

EFFECTS OF GENETICALLY MODIFIED EUCALYPT POLLEN ON *Scaptotrigona bipunctata* (MELIPONINI) AND *Apis mellifera* (APINI) BEES

Flowering mass of eucalypts trees are attractive to the bees for collection of floral resources, mainly pollen and nectar. The worldwide distribution of *Eucalyptus* species and the novel generation of genetically modified trees all over the world, especially in Brazil and USA, emphasize the need of information concerning environmental risks, including potential effects on bees. Since the transgenic events are expressed as proteins, the pollen becomes the main route of exposure of transgenic proteins to the bees. The present study aimed to assess the effects of ingestion of pollen from forest areas with genetically modified (GM) eucalypt trees on development of *Apis mellifera* and *Scaptotrigona bipunctata*. For this, we assessed the synchronism on development of *Apis mellifera*, the morphometry of *A. mellifera* and *S. bipunctata* on pupal phase and the weight of *S. bipunctata* immatures. **Trials with *A. mellifera*:** in February and March 2011, the study was carried out in an experimental area with GM eucalypt and in a commercial area with conventional trees, without GM eucalypt. *A. mellifera* colonies were placed in triplicates in the center of experimental area, 400 m, 1.000 m and 85 Km away from area. Brood combs were harvested and the pupae were assessed regarding to the developmental stage, head width (HW) and intertegular distance (ID). **Trials with *S. bipunctata*:** under controlled conditions, micro colonies of *S. bipunctata* were subjected to feeding with GM and non-GM eucalypt pollen. The pollen was obtained from pollen collectors placed in three colonies of *A. mellifera* established in the experimental area and three colonies in the commercial area. We collected from 10 to 15 pupae/comb, which the weight, HW and ID measurements were obtained. In *A. mellifera* was verified similarity in proportions of the different developmental stages in all areas. We infer regularity of queens laying of *A. mellifera* on period, because the spatial-temporal pattern of laying occurred as expected. Regarding to morphometric analysis, differences were observed. The ID mean of pupae from 1.000 m differed from the ID mean found for pupae from colonies in the experimental area and 85 Km away. Differences were also observed in pupae from hives from 400m and 85 Km away. We consider the differences showed as expected, because *A. mellifera* represent hybrid populations in Brazil, with phenotypic expression among the individuals. Since the differences verified are not progressively related to the distance from the experimental area, the hypothesis of changes caused by ingestion of GM pollen is discarded. Regarding to the morphometric characteristics obtained for *S. bipunctata*, differences were observed. Nevertheless, the variation coefficient for all micro colonies was lower than 10%, indicating homogeneous groups. Therefore, these differences may be due to of the genetic variation among the populations. The weight analysis of *S. bipunctata* pupae indicated similarity, which showed a mean of 0.0212 g (SD+-0.0028 g) when fed with GM eucalypt pollen and 0.0204 g (SD+-0.0019 g) when fed with non-GM eucalypt pollen. According to the results, the similarity found in the weight of pupae fed under both treatments suggest that the effect of GM pollen was similar to its non-GM isolínea, cause no harm to the bees. Thus, the hypothesis of changes caused by GM pollen consumption is discarded.

APRESENTAÇÃO

A eucaliptocultura apresenta-se no Brasil como a principal atividade silvícola, com cerca de 5.102.030 ha de área plantada. A crescente demanda de setores industriais, como o de papel e celulose, tem levado pesquisadores e demais profissionais da área a buscarem por métodos alternativos para o aumento da produtividade florestal. Para tanto, a biotecnologia tornou-se uma ferramenta essencial, pois através do melhoramento genético de espécies arbóreas, características como tolerância a estresses abióticos (seca, frio) e bióticos (insetos-praga, plantas daninhas) e qualidade da madeira e da fibra podem ser adquiridas. No entanto, para a liberação comercial de cultivos geneticamente modificados, faz-se necessário a realização de testes que avaliem os potenciais impactos destas culturas ao ambiente. Desta forma, testes de avaliação de risco são realizados com organismos não-alvo, incluindo os insetos polinizadores. Dentre estes destacamos as abelhas, que são atraídas pelas flores de eucalipto para a coleta de recursos alimentares como néctar e pólen. As abelhas são importantes ecológica e economicamente por seus serviços de polinização, contribuído tanto para a produção de alimentos como para a manutenção do equilíbrio ambiental a partir da reprodução de espécies vegetais. Tendo em vista isso, este trabalho busca avaliar os efeitos da ingestão de pólen proveniente de áreas com árvores de eucalipto geneticamente modificado em duas espécies de abelhas: *Apis mellifera*, considerada modelo experimental em análises de risco; e *Scaptotrigona bipunctata*, espécie nativa com ampla distribuição geográfica e manejada comercialmente para fins de produção de mel e polinização agrícola.

Dois artigos foram produzidos a partir dos resultados dessa pesquisa e compõem esta dissertação sob a forma de capítulos descritos a seguir.

Capítulo I: **Effects of genetically engineered eucalypts on the pattern of spatial-temporal-morphological development of** *Apis mellifera.* Neste capítulo é apresentado o primeiro estudo de avaliação dos efeitos de uma espécie arbórea transgênica em *Apis mellifera* o qual será submetido como artigo à revista Ecotoxicology and Enviromental Safety.

Capítulo II: **Potenciais efeitos da ingestão de pólen proveniente de área com árvores de eucalipto geneticamente modificado em** *Scaptotrigona bipunctata* (Apidae: Meliponini)**.** Este estudo constitui a avaliação dos potenciais efeitos de uma espécie de eucalipto transgênico em abelhas sociais nativas. Este capítulo será

submetido como artigo à revista Transgenic Research. Os dois manuscritos foram estruturados de acordo com as normas estabelecidas com os respectivos periódicos científicos.

CAPÍTULO I

EFFECTS OF GENETICALLY ENGINEERED EUCALYPTS ON THE PATTERN OF SPATIAL-TEMPORAL-MORPHOLOGICAL DEVELOPMENT OF *Apis mellifera*

EFFECTS OF GENETICALLY ENGINEERED EUCALYPTS ON THE PATTERN OF SPATIAL-TEMPORAL-MORPHOLOGICAL DEVELOPMENT OF *Apis mellifera*

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Abstract

The flowers of eucalypt trees are intensely sought by bees in order to collect nectar and pollen. The wide distribution of the *Eucalyptus* genus and the recent generation of genetically engineered (GE) trees around the world, especially in Brazil and the US, emphasize the need of information on environmental risks, including possible effects on bees. The present study aimed to evaluate the effects of interactions between GE eucalypt trees and *Apis mellifera* on the pattern of spatial-temporal-morphological development of bee immature stages. The study was conducted in a field trial of GE and surrounding areas of non-GE *E. grandis* x *E. urophylla* hybrid trees located in the state of São Paulo, Brazil, during February and March 2011. Hives were placed in triplicates in the center of a 1.3-hectare GE eucalypt field trial and at different distances (400, 1,000 and 85,000 m) from the trial. Brood combs were collected and the developmental phase of pupa was evaluated based on measurements of head width (HW) and intertegular distance (ID). The number and distribution of immatures per comb allowed us to first infer that queen oviposition was regular because of the equivalent temporal and spatial patterns of laying eggs, occurring as expected. Similarities among the proportions of the different developmental phases of larvae were observed. The variation in HW and ID values found in bees from the different locations were not related to the presence of GE trees but by the fact that honey bees represent hybrid populations with phenotypic variation among individuals. The differences verified were not progressively related to the distances from the GE experimental area and, therefore, the hypothesis of changes caused by ingestion of GE pollen was discarded.

Key-words: *Eucalyptus*, risk assessment, morphometry, synchronism of development, genetically engineered plant, honey bee.

Introduction

Genetic engineering is being applied to numerous crops especially corn, soybean, oilseed rape and cotton (Park et al. 2011). So far, genetically engineered (GE) crops have been widely accepted in vast commercial plantations around the world (James 2012). The release of GE crops in the environment has raised concerns about the possible impacts on non-target organisms (Romeis et al. 2008; Wolt et al. 2010; Hilbeck et al. 2011; Romeis et al. 2011). More recently, biotechnology has allowed the generation of GE forest trees intended to supply the growing demands of timber, cellulose, paper and biofuel (Strauss et al. 2009; Harfouche et al. 2011; Fladung et al. 2012; Meilan et al. 2012). Trees of the *Eucalyptus* genus have been targeted by intense genetic breeding programs, including genetic engineering, in Brazil, USA and other countries (Grattapaglia and Kirst 2008; Harfouche et al. 2011), aiming at yield increase and improvement of wood quality.

The uncertainties related to possible negative effects caused by GE plants on non-target organisms, including pollinator insects, have developed the need for risk assessment tests. Honey bees (*Apis mellifera* L.) are among the pollinator insects considered in environmental risk assessments, and assume special importance because they are strongly attracted by flowers as their food source and, therefore, have an equal relevance to both biosystems and the economy (Potts et al. 2010; Winston 1987). The flowers of *Eucalyptus* trees are very attractive to insects, and bees are the main representative of their associated fauna (Horskins and Turner 1999; Nicolson 2011). In this context, bees may be considered as important indicators of possible disturbances caused by the introduction of GE plants into the environment. Due to its wide distribution and its ecological and economical value, *A. mellifera* has been used as an

experimental model in risk assessments of GE organisms (Malone et al. 2001; Malone and Pham-Delègue 2001; Babendreier et al. 2004; Rose et al. 2007; Duan et al. 2008).

Pollen is essential for the development of bees, since it serves as their main protein source, initially as larval food but also used along the adult life (Crailsheim et al. 1992; Zerbo et al. 2001; Brodschneider and Crailsheim 2010). Nectar is equally important since it represents the main carbohydrate source for bees. In fact, nectar represents the main raw material for the elaboration of honey (Doner 1977; Winston 1987). The most immediate novelty produced by GE plants is a recombinant protein. Proteins are detected in very low amounts in nectar (Peumans et al. 1997; Malone and Burgess 2009), so the potential adverse effects on bees caused by GE trees may occur by the ingestion of pollen, since its protein composition can range from 2.5 to 65% protein (Roulston 2000; Malone and Pham-Delègue 2001; Brodschneider and Crailsheim 2010). However, information regarding the levels of recombinant proteins in pollen is rarely presented, and alternative methods have been developed in order to test the potential adverse effects of GE plants on bees. As an example, risk assessments have been based on feeding purified recombinant proteins to evaluate the direct effects on the development of individual bees or other insects (Malone 2002; Brødsgaard et al. 2003; Romeis et al 2011). *In vitro* experiments have shown the safety or equivalence of recombinant proteins to non-recombinant ones based on short-term results (Lima et al. 2010; Romeis et al. 2011). Although quite relevant, these assays represent "worse scenarios", and are valid only for the pure recombinant protein and cannot be related to the real availability of recombinant proteins that bees would have contact with in the environment (Malone and Pham-Delègue 2001). It is important to consider that, besides the recombinant protein itself, effects may be caused by intended, altered metabolites resulting from transgene expression, or by unintended products that are the result of

interactions among genes or pleiotropy (Uberlacker et al. 1996). In addition, *in vitro* assays of pure recombinant proteins directly on bee immatures ignore the ecological impact on bee colony, since such assays are not considering the social interactions in hives.

Modifications in the chemical composition of pollen may disturb the behavior (such as the egg laying activity of queens) of individual bees in hives (Sokol 1996; Heather et al. 2006). Under normal conditions, the brood provisioned in cells near to each other has similar ages and therefore, the same stage of larval development, although small temporal differences may occur (Winston 1987). Phenotypic changes in morphology can be measured as well, indicating possible alterations during the ontogeny of individuals (Silva et al. 2006). Based on these assumptions, this work aimed to assess the potential impacts of GE pollen ingestion on the egg laying activity of queen bees and the development of *A. mellifera* immatures from hives with free access to GE and non-GE *Eucalyptus* trees in the field.

Material and Methods

GE eucalypts, field test, non-GE control areas, and honey bees

The study was conducted in two forest plantations of *Eucalyptus grandis x E. urophylla* hybrid trees belonging to FuturaGene Ltd. A 1.3 hectare (ha) field test of GE eucalypts was located in Angatuba (23°29’28,20’’ S, 48°35’59,4’’ O) and a 600-ha commercial plantation of non-GE eucalypts was located in Alambari (23°31’44,83’’ S, 47°50’53,72’’ O). Both municipalities belong to the state of São Paulo, southeast Brazil. *E. grandis x E. urophylla* leaves were genetically transformed via the *Agrobacterium tumefaciens* method essentially as described by González et al. (2002). Transgene constructs and T-DNA regions of the binary vectors employed are detailed in Figure 1.

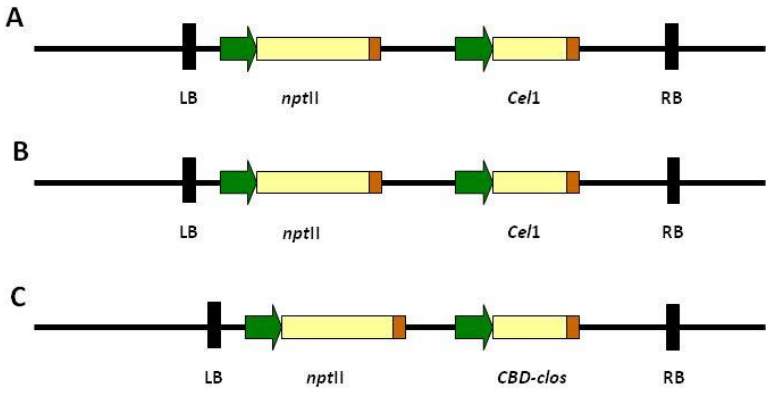

Figure 1. Transferred-DNA regions of binary plasmids pMH42 (A), pConstitutive-CEL1 (B) and pMH11 (C) employed to genetically transform *E. grandis x E. urophylla* by *A. tumefaciens*. LB and RB, T-DNA left and right borders; *npt*II, neomycin phosphotransferase gene; *Cel*1, gene encoding endo-1,4-β-glucanase, EC 3.2.1.4 (Shani et al., 1997; 2006); *CBD-clos*; gene encoding the cellulose-binding protein A, non glycosylated form from *Clostridium cellulovorans* (Shoseyov et al., 1992). Green arrows represent promoter regions and orange rectangles represent terminator sequences. The *Cel*1 gene in A and B is under the regulation of different constitutive, strong promoters while the *CBD-clos* gene in C is regulated by a tissue-specific promoter.

Around 1,150 clonal GE plantlets (40-50 cm high) were randomly planted in the 1.3-ha GE test field in February 2007, with a 3 m spacing between neighbor lines and 2 m spacing between neighbor trees. A total of 50 non-GE, control plantlets of the same genetic background and age were also planted inside the area, representing 4.2% of the trees. The 1.3-ha test field was surrounded by two lines of non-GE control trees and by 300 m of grass field (buffer zone). Commercial plantations of non-GE eucalypts surrounded the grass field. The experimental design of the test field is represented in Figure 2.

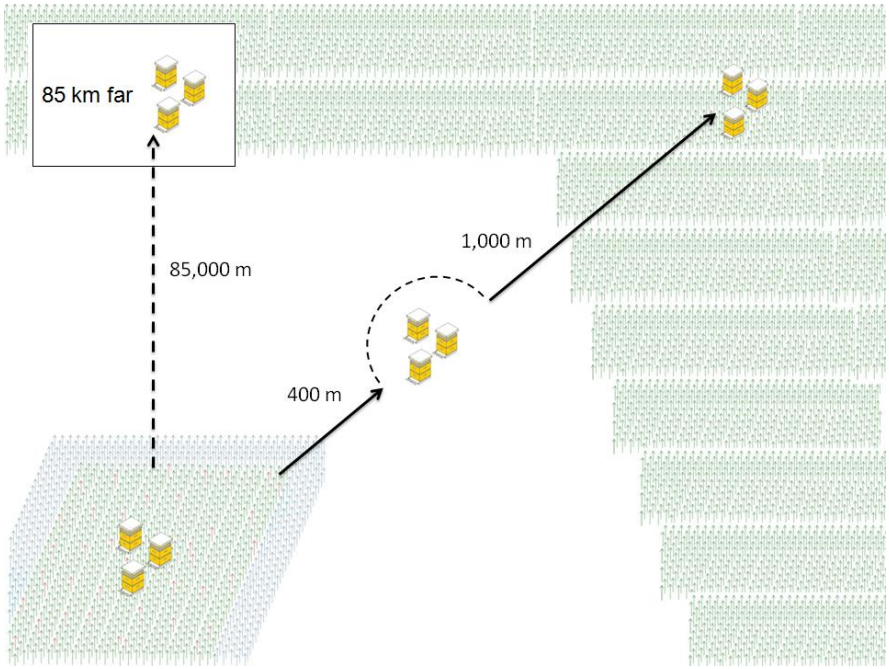

Figure 2. Test field of GE eucalypts and surrounding areas. The 1.3-ha test field of GE eucalypt is represented in the bottom-left of the figure. Trees are represented by small vertical green (GE), red or blue (non-GE) arrows. Surrounding the test field is a 300 m grass field and a commercial plantation of non-GE eucalypt trees (small vertical green arrows). Honey bee hives were positioned as indicated. Note that hives positioned 85 km far from the test field are at a distance not drawn to scale (dotted line).

Data were collected from 12 wooden hives (Langstroth model) of *A. mellifera*. Queenright beehive bodies were mounted with queen excluder meshes installed between hive bodies and covers as well as at the entrances. Beehives were installed in eucalypt fields in January 12th 2011 when trees were 4-years-old (after planting), during the main blooming period. Three beehives were positioned in the center of the GE eucalypt test field, 3 m apart from each other (see Figure 2). Groups of 3 beehives were similarly positioned at 400 m from the test field (grass field) at 1,000 m (non-GE eucalypt commercial plantation), and 85,000 m from the test field (non-GE eucalypt commercial plantation), exceeding the radius of bee flight, which is known to be around 10 km (Visscher and Seeley 1982). After placing beehives in the study sites, frames with

honey and pollen from the edges were removed and two new frames with laminated wax were positioned in the center of all hives. Comb samples with operculated broods were collected from hives every two-weeks (March 2nd, 16th and 30th 2011) according to Table 1. Each comb sample consisted of 12 x 12 brood cells. Comb samples were stored in plastic sealed boxes and immediately analyzed.

Table 1. Sampling of bee broods according to the experimental areas, beehives and dates of harvesting. Signs ● and X indicate if brood was harvested or not, respectively.

Location	Beehives	Days of sampling (March 2011)		
		2nd	16th	30th
GE	1	X	X	●
	2	●	●	X
	3	●	●	●
400 m	1	●	●	●
	2	●	●	●
	3	●	●	●
1,000 m	1	●	●	●
	2	●	●	●
	3	●	●	●
85,000 m	1	●	●	●
	2	●	●	●
	3	●	●	●

Evaluation of the synchronism of *A. mellifera* larval development

Under stereoscopic microscope (OLYMPUS, SZ61), 144 cells of each brood comb sample were opened and the developmental phase of each immature was identified, considering the spatial position in the comb (Figure 3). The spatial distribution of immatures was analyzed considering three concentric areas (Figure 4) with 48 cells each one, adapted from Moo-Vale et al. (2004). The development of immatures was classified in prepupa (PP), pupa with white eyes (P1), pupa with pigmented eyes (P2) and imago (Im; Figure 3). Empty cells in samples were also considered. The proportions among the different phases of development were compared, applying the non parametric Kruskal-Wallis (SPSS 17.0) test of variance with a significance of 5%.

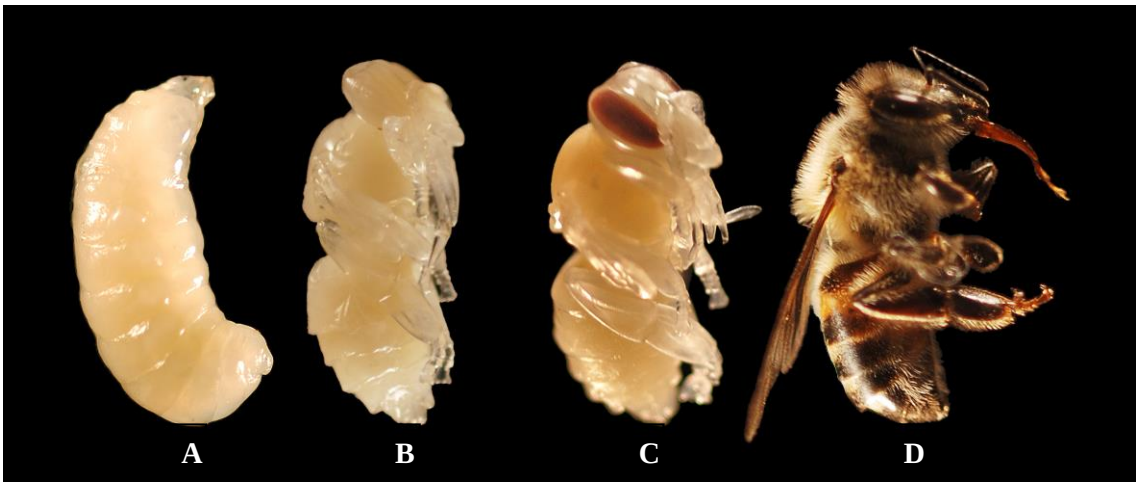

Figure 3. Stages identified during the development of *A. mellifera*. Prepupa (A), pupa with white eyes (B), pupa with pigmented eyes (C) and imago (D).

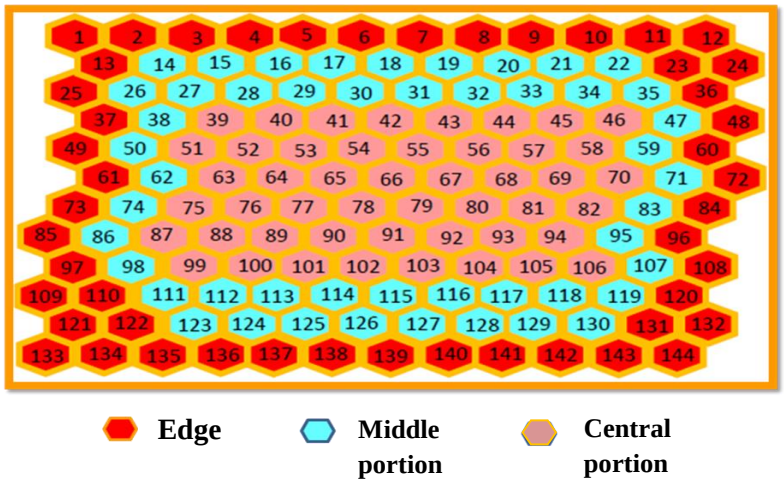

Figure 4. Subsamples established in combs in order to define the synchronicity of development of immature *A. mellifera* according to their spatial distribution. Red colored hexagons represent edge cells, blue colored are cells at the middle portion, and pink hexagons represent cells in the central portion of the sample. Each subsample portion is therefore composed of 48 cells.

Morphometric analysis of immatures of *A. mellifera*

Morphometric measurements were performed in 35 randomly chosen pupa per comb. With a digital caliper (MITUTOYO IP66), the head width (HW) and the intertegular distance (ID) of *A. mellifera* in pupal stage were evaluated (Bullock et al. 1999; Bosch and Vicens 2002; Gilley et al. 2003; Kuhn-Neto et al. 2009). Resulting data were submitted to a multivariate ANOVA test according to the General Linear Model (GLM; *post hoc* test of Tukey; SPSS 17.0) with a significance of 5% in order to allow comparisons of pupae measures obtained from hives at the four distances from the GE test field.

Results

Synchronism of *A. mellifera* development

The present study aimed to evaluate possible effects of GE eucalypt pollen on the development of *A. mellifera* immatures in open field tests. To do so, bee hives were positioned in experimental areas of GE eucalypt and at different distances from it, as shown in Figure 2. Samples of broods were gathered and the developmental phase of each immature was evaluated. With such approach we intended to first check possible alterations in the synchronism of development due to the presence (or not) of GE pollen and nectar in beehives located in the GE test field compared to beehives positioned at increasing distances.

An evident similarity among the proportions of different immature developmental stages was observed in combs from hives located in all study areas based on a Kruskal Wallis test of variance ($p > 0.05$), as shown in Table 2. A predominance of the P2 phase of development in all combs sampled was observed while Im was the developmental stage least represented.

Table 2. Representative mean percentage of *A. mellifera* developmental stages observed according to subsampling in central, middle and edge portions of each comb. Combs were taken from hives located inside the GE eucalypt test field (GE), and at distances of 400, 1,000 and 85,000 m far. Number of combs and number of immatures evaluated are indicated. PP, prepupa; P1, pupa with white eyes; P2, pupa with pigmented eyes; Im, imago.

Location (combs) (immatures)	Comb portion	Developmental stage				
		PP	P1	P2	Im	Empty
GE (6) (864)	Edge	6.59±0.13	17.71±0.24	64.58±0.27	4.17±0.06	6.94±0.04
	Middle	2.08±0.03	21.52±0.35	65.97±0.34	4.86±0.07	5.55±0.03
	Central	1.04±0.01	18.73±0.30	69.83±0.30	4.16±0.06	6.22±0.01
400 m (9) (1,296)	Edge	10.41±0.23	14.81±0.23	65.97±0.38	0.69±0.02	8.10±0.05
	Middle	10.41±0.26	13.88±0.26	66.89±0.38	0.92±0.02	7.87±0.06
	Central	11.80±0.31	9.25±0.27	72.40±0.41	0.23±0.01	6.29±0.04
1,000 m (9) (1,296)	Edge	18.05±0.30	18.05±0.20	54.62±0.32	0.00±0.00	9.25±0.04
	Middle	13.88±0.31	16.66±0.23	61.11±0.35	0.00±0.00	8.33±0.02
	Central	13.03±0.30	18.77±0.31	56.76±0.40	0.00±0.00	11.41±0.04
85,000 m (9) (1,296)	Edge	12.96±0.27	3.93±0.07	67.36±0.26	2.77±0.04	12.96±0.06
	Middle	13.68±0.29	3.24±0.07	70.12±0.27	3.23±0.04	9.71±0.06
	Central	11.57±0.29	1.38±0.03	75.00±0.30	3.01±0.05	9.03±0.06

The similarity in developmental synchronism of larvae belonging to beehives located within or outside the GE eucalypt test field allowed us to conclude that, besides the absence of effects directly on larval development, queen bee oviposition activity was also not affected by the transgenes or the resulting phenotype of the GE trees.

Morphometric analysis of *A. mellifera*

Morphological changes in bees and other insects as the result of altered patterns of ontogeny are known to be determined by genetic or environmental factors including food availability, quality or chemical composition (Oldroyd et al. 1991; Silva et al. 2006). In order to check if the GE eucalypt trees or derived pollen and nectar were determining morphological alterations in bees, we evaluated two morphometric parameters in pupa from all beehives: head width (HW) and intertegular distance (ID).

Measurements of HW resulted in averages of 3.87±0.22 mm, 3.91±0.15 mm, 3,91±0,21 mm, and 3.86±0.21 mm in pupa from beehives respectively located within

the GE eucalypt field test, and at distances of 400, 1,000, and 85,000 m. As shown in Figure 5, no differences in averages of pupa HW were statistically significant based on a multivariate ANOVA test according to the GLM (*post hoc* test of Tukey; $p > 0.05$).

The ID values obtained from pupa of beehives located inside the GE eucalypt test field resulted in an average of 3.19±0.19 mm. Pupa from increasingly distant beehives exhibited ID averages of 3.16±0.18 mm (400 m), 3.12±0.18 mm (1,000 m), and 3.21±0.27mm (85,000 m). The average pupa ID from beehives located at 1,000 m was statistically distinguishable from values found in pupa from beehives at the GE test field and at 85,000 m (Figure 5). A statistical difference in ID averages was also observed in pupa from beehives located at 400 and 85,000 m far from the GE test field. It was clear that differences in the ID morphometric parameter were not determined by the GE eucalypt trees since no differences were observed between pupa from beehives located inside the GE test field and in the control area at 85,000 m distance.

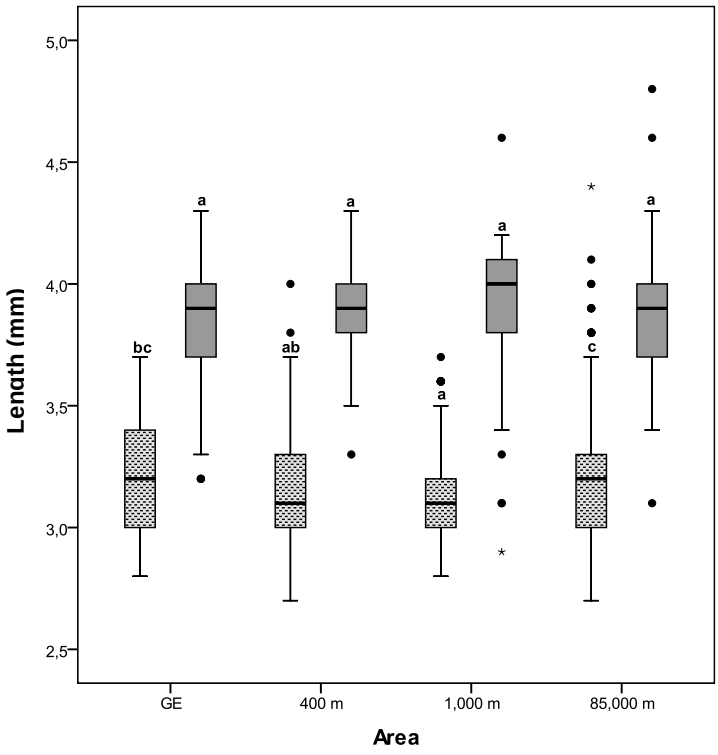

Figure 5. Average values derived from morphometric analysis of *A. mellifera* pupa from beehives placed inside the GE eucalypt test field (GE) and at increasing distances (400, 1,000, and 85,000 m). Solid rectangles, Head Width (HW); dotted rectangles, Intertegular Distance (ID). Central lines indicate median values; black points and asterisks represented outliers and extremes, respectively; similar means are represented by same letters (ANOVA GLM test, with Tukey *post hoc* for $p > 0.05$).

Discussion

The strong attractiveness of eucalypt flowers to honey bees and other insects highlights the need for GE trees risk assessment studies in field trials as well as under containment (greenhouses or laboratorial facilities). While such approaches have been more extensively conducted with GE crops like corn, soybean, canola and cotton expressing bacterial or fungal genes for herbicide tolerance or insect resistance, little has been done with transgenic trees, especially eucalypts (Romeis et al. 2008; Wolt et al. 2010; Harfouche et al. 2011; Hilbeck et al. 2011; Romeis et al. 2011).

The *E. grandis x E. urophylla* hybrid GE trees under analysis were able to express transgene combinations of either *npt*II and *Cel*1 or *npt*II and *CBD-clos* (Figure 1). The *npt*II gene, originally from *Escherichia coli*, encodes the enzyme responsible for the resistance to neomycin and other aminoglycoside antibiotics. In GE eucalypts as well as in all other GE plants, the presence of the *npt*II gene allows the selection and regeneration of transformed tissues and plants during *in vitro* laboratorial steps (Miki and McHugh 2004). A large body of scientific evidences have shown that the *npt*II and its encoded enzyme, as well as other selectable marker genes and proteins, are safe to human and animal health as well as to the environment (reviewed in Miki and McHugh 2004; EFSA, 2007; Ramessar et al., 2007).

The *Cel*1 gene from *Arabidopsis thaliana* encodes a endo-1,4-β-glucanase known to hydrolyze polysaccharides of plant cell walls possessing 1,4-β-glucan. The *CBD-clos* gene from *Clostridium cellulovorans* encodes a cellulose-binding domain

protein that plays essential roles in the mechanism of cellulose degradation and, likewise, has the potential to modify cellulose-containing materials. Both proteins are therefore fundamental for plant cell growth acting in the partial degradation of cell walls (Levy et al., 2002; Shoseyov et al., 2006). These genes were employed by FuturaGene Ltd. in order to improve the growth rate and wood quality of *E. grandis x E. urophylla* trees. It is important to mention that in the 1.3 ha GE test field, 96% of the trees were transgenic for *npt*II, 66% of them were transgenic for *Cel*1, and 33% of the trees harbored the *CBD-clos* transgene. Results concerning safety assessments of *Cel1* and *CBD-clos* genes in GE plants are not yet available, motivating the present work.

Although it is not expected a direct negative effect of the three transgene expressions on the level or quality of proteins and nectar in GE eucalypt pollen and, consequently, the modification of honey bee development or behavior when feeding on such GE pollen, the over- or novel expression of genes related to cellulose metabolism could affect the synthesis or degradation of other relevant metabolites like alkaloids, phenylpropanoids and terpenes. These (and other) secondary metabolites have in turn relevant ecological functions (Theis and Lerdau, 2003). The indirect alteration of their metabolisms determined by transgene expression could for instance reflect on the attractiveness or repellence of insects, or determine different levels of toxicity.

When evaluating the effects of pollen or nectar on bees, it is important to consider all phases of insect development, since bees keep close contact with floral resources during all ontogeny (Winston 1987). Notwithstanding, studies focused on immature phases of development were pointed out to be more effective to evidence toxicity since it is during the larval phase that bees consume the highest amounts of pollen (Malone 2002; Brodsgaard et al. 2002; Hendriksma et al. 2012).

Results presented here indicated that *A. mellifera* queens exposed to four-year-old GE eucalypts in blossom planted in a 1.3 ha area did not exhibit differences in the pattern of egg laying activity compared to queen bees from hives located at distances of up to 85 km from the GE field test. Additionally, the analysis of egg/immature distribution in three concentric areas of broods, employed in the present study, showed that all individuals presented close synchronism in development in all areas considered. The egg laying behavior of *A. mellifera* is taken to evidence toxic effects of different compounds, especially insecticides. Haarmann et al. (2002) for instance found that queens treated with the organophosphorus coumaphos insecticide exhibited physical abnormalities and atypical behavior. Experiments developed by Sokol (1996) showed that the use of the pyrethroid insecticide fluvalinate caused serious disturbances in bee hives, altering dramatically the egg laying activity and determining queen losses. The present work allowed us to infer that queen oviposition was regular considering temporal and spatial patterns of egg laying independently of the presence or absence of GE trees harboring the three mentioned genes.

Morphometric measurements of head widths (HW) and intertegular distances (ID) of *A. mellifera* pupa demonstrated that bees kept in the GE-test field were indistinct from those positioned at 400 m and at 85 km far from the GE-test field. While HW values were also equivalent in bees kept at 1 km far, these bees exhibited shorter ID than those at the GE-test field ant those at the control area, at 85 km distance. We thus considered that this difference was justified by the fact that honey bees in Brazil represent hybrid populations with small differences in the size of individuals, coloring pattern, resistance to pathogens, and even in population sizes (Nunes et al. 2012; Lobo et al. 1989). Genetic differences between bees from different hives are therefore expected since social insects are generated from multiple mating (Cole 1983; Couvillon

et al. 2010). Thereby, since differences verified were not progressively related to the distance from the GE experimental area, the hypothesis of changes caused by the ingestion of GE pollen was discarded.

During the past years, essential global insect pollinators such as *A. mellifera* are suffering severe declines in their populations (Biesmeijer et al. 2006; Pettis and Delaplane 2010; Potts et al. 2010). In this context, phenomena currently known as 'Colony Collapse Disorder' (CCD) or massive disappearance of bees are under investigation. There are still no plausible explanations for such disorders but it is known that the sanity of bee hives are affected by environmental adversities such as temperature, presence of enemies, malnutrition, and the irrational use of pesticides (Wu et al. 2011). It has also been proposed that toxic substances from GE plants, especially those encoding insecticide proteins, may disturb relevant pollinator insect populations like honey bees (Romeis et al. 2008). In the present work we showed that GE eucalypt trees harboring the *npt*II marker gene and genes related to cellulose modulation did not determine disturbances either on the egg laying behavior of honey bee queens or on the development of immatures.

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Short title: GE eucalypt effects on *Apis mellifera*

CAPÍTULO II

POTENCIAIS EFEITOS DA INGESTÃO DE PÓLEN DE EUCALIPTO GENETICAMENTE MODIFICADO EM *Scaptotrigona bipunctata* (APIDAE: MELIPONINI)

POTENCIAIS EFEITOS DA INGESTÃO DE PÓLEN DE EUCALIPTO GENETICAMENTE MODIFICADO EM *Scaptotrigona bipunctata* (APIDAE: MELIPONINI)

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RESUMO

No Brasil, árvores de espécies de *Eucalyptus* são alvo de experimentos com transgenias e por este motivo testes de análise de risco a organismos não-alvo são indicados com vistas a sua liberação comercial. Considerando que as abelhas sociais apreciam as florações em massa destas árvores para a coleta de recursos alimentares, espécies do grupo devem ser adotadas nas análises de risco ambiental. Uma vez que o pólen é a principal via de contato de proteínas GM ás abelhas, o presente estudo testou o efeito da ingestão de pólen de eucalipto GM por *Scaptotrigona bipunctata* na morfometria e na massa corporal de operárias em estágio pupal. A obtenção de amostras de pólen foi realizada com armadilhas na entrada de colmeias de *Apis mellifera*, dispostas em horto experimental com eucalipto GM e em horto convencional com plantas da mesma isolínea não-GM. Em laboratório, submeteu-se minicolônias de *S. bipunctata* confinadas a alimentação com pólen oriundo da área experimental com eucalipto GM e da área convencional, livre de eucalipto GM. As pupas derivadas dos dois tratamentos foram coletadas e avaliadas quanto a massa corporal, largura da cabeça e distância intertegular. Observou-se semelhança na massa corporal das pupas, em ambos os tratamentos, a qual sugere que o efeito do pólen manteve-se semelhante a sua isolínea não-GM, sem prejuízo às abelhas. Com relação a morfometria, as diferenças encontradas podem ser reflexo da variação genética entre populações, uma vez que o coeficiente de variação indicou grupos homogêneos. Portanto, descarta-se a hipótese de alterações ocasionadas por consumo de pólen GM.

Palavras-chave: Eucaliptocultura, abelhas sem-ferrão, transgenia, morfometria, minicolônias

Introdução

O Brasil possui posição de destaque mundial na produtividade de suas florestas plantadas de eucalipto, bem como na produção de celulose, papel e madeira. A eucaliptocultura é a principal atividade silvícola no País, com uma área de 5.102.030 ha destinados ao cultivo de plantas do gênero *Eucalyptus* (ABRAF 2013). À semelhança de culturas agrícolas anuais como soja, milho, arroz e canola, recentemente o eucalipto vem sendo alvo de experimentos com transgenia (Grattapaglia e Kirst 2008; Harfouche et al. 2011). Com vistas ao aumento da produtividade dos maciços florestais de eucalipto, os transgenes arbóreos estão sendo gerados com características como menor teor de lignina, maior produção de celulose, crescimento acelerado e tolerância ao frio e a seca (Pasquali e Bodanese-Zanettini 2007).

Para a liberação comercial de culturas geneticamente modificadas (GM) exige-se a verificação da inocuidade destas culturas no que diz respeito aos organismos não-alvo. A avaliação dos potenciais efeitos de tais culturas implica em análises de risco ambiental, conduzidas usualmente em insetos polinizadores (Conner 2006), os quais apreciam as flores de eucalipto para a coleta de néctar e pólen como recursos alimentares (Kleinert-Giovanini e Imperatriz-Fonseca 1987; Ramalho et al. 1990; Wilms e Wiechers 1997; Horskins e Turner 1999; Marchini e Moreti 2003; Antonini et al. 2006; Kajobe 2007, Hilgert-Moreira et al. 2014).

Os insetos são considerados o principal e mais diverso grupo de polinizadores (Roubik 1989; Ollerton et al. 2011), e dentre estes podemos destacar as abelhas, as quais representam cerca de 40% do grupo (Biesmeijer e Slaa 2004). Importantes ecológica e economicamente por seus serviços de polinização, as abelhas são consideradas organismos chave em avaliações de risco de cultivos GM (Kevan 1999). *Apis mellifera* é o modelo experimental mais utilizado em experimentos com transgenias (Malone et al. 2001; Malone e Pham-Delègue 2001; Rose et al. 2007; Duan et al. 2008) devido ao seu alto valor econômico e ampla distribuição geográfica (Huryn 1997; Goulson 2003). No entanto, a exemplo de *A. mellifera*, as abelhas nativas também devem ser incluídas em análises de risco ambiental, uma vez que são responsáveis pela manutenção de ecossistemas (Slaa et al. 2006). No Brasil, a utilização de abelhas nativas como alternativa para a realização de estudos de risco ambiental deve ser considerada, especialmente em virtude da alta diversidade destes organismos (Lima et al. 2008). Esta situação pode ser ilustrada com *Scaptotrigona bipunctata* (Apidae: Meliponini), espécie rústica, a qual nidifica em cavidades pré-existentes, predominantemente em ocos de árvores (Nogueira-Neto 1970). Assim como *A. mellifera*, *S. bipunctata* possui colônias populosas e perenes, as quais exploraram um amplo espectro de fontes botânicas ao longo do ano, caracterizando seu hábito alimentar generalista (Ramalho et al. 2007).

O pólen é a principal fonte proteica para as abelhas, podendo chegar até 60 % de proteínas em sua composição (Roulston et al. 2000). Por ser um componente indispensável durante o desenvolvimento larval (Zerbo et al. 2001), quaisquer alterações na composição química do pólen poderiam afetar, por exemplo, o desenvolvimento das glândulas hipofaringeanas, a morfologia corporal e a longevidade das abelhas (Brodschneider e Crailsheim 2010). Os eventos geneticamente modificados em plantas são expressos sob a forma de proteínas e portanto, o pólen é considerado a principal via de contato de proteínas GM com as abelhas (Babendreier et al. 2004).

Babendreier e colaboradores (2005) avaliaram o efeito do pólen de milho transgênico e sua isolínea não-transgênica no desenvolvimento das glândulas hipofaringeanas de *A. mellifera*. O tamanho e peso das glândulas mostram-se semelhantes para ambos os tratamentos, inferindo a ausência de efeitos negativos provenientes da linhagem transgênica. Da mesma forma, Geng e colaboradores (2012) não observaram anormalidades na diversidade da flora intestinal de operárias recém emergidas de *Apis mellifera lingustica* quando alimentadas com pólen transgênico de milho. Huang e colaboradores (2004) testaram os efeitos da ingestão do pólen de canola transgênica por larvas e adultos de *A. mellifera* e não observaram diferenças na mortalidade e peso dos indivíduos, quando comparados com aqueles que ingeriram pólen de canola não transgênica e orgânica.

A quantificação de proteínas GM no pólen é um processo considerado complexo, uma vez que são necessários marcadores específicos para sua identificação no conteúdo celular. Em vista disso, métodos alternativos vem sendo desenvolvidos, os quais fazem uso de proteínas GM purificadas em experimentos com abelhas, similares aos testes realizados com inseticidas (Malone e Pham-Delègue 2001). Embora estudos em laboratório detectem efeitos adversos a partir de testes *in vitro* com proteínas purificadas (Brødsgaard et al. 2003; Malone 2002; Babendreier et al. 2008), permanece complexa a compreensão dos impactos ecológicos em colmeias de abelhas (Huang et al. 2004). Tais testes eliminam as interações sociais das colônias, limitando a compreensão dos impactos ocasionados. Além disso, estes testes desconsideram a possibilidade de efeitos pleiotrópicos ou de interação entre genes, os quais podem promover efeitos indiretos às abelhas (Uberlacker et al. 1996). Tais efeitos podem refletir na fisiologia da planta, levando a modificações na atratividade das flores e alterações no valor nutritivo de produtos como néctar e pólen (Malone e Pham-Delègue 2001).

Segundo Malone (2002), os potenciais efeitos de todo e qualquer produto proveniente de plantas geneticamente modificadas devem ser avaliados mesmo que a proteína recombinante não seja expressa diretamente no produto testado. Desta forma, considerando que alterações químicas no pólen podem modificar seu valor nutricional às abelhas e com isso acarretar distúrbios na ontogenia dos indivíduos, o presente estudo testou o efeito da ingestão de pólen de *Eucalyptus* spp. GM por *Scaptotrigona bipunctata* na morfometria e na massa corporal de operárias em estágio pupal de desenvolvimento.

Materiais e Métodos

Estabelecimento de minicolônias de *Scaptotrigona bipunctata*

Seis minicolônias foram originadas a partir de colônias-matrizes de *S. bipunctata* mantidas no meliponário da Pontifícia Universidade Católica do Rio Grande do Sul, localizado no município de Porto Alegre, sul do Brasil. Para o estabelecimento das minicolônias, transferiu-se de uma colônia-matriz a rainha, dois favos de cria em fase larval e pré-pupal, cerca de 250 operárias e material de construção (adaptado de Menezes e Imperatriz-Fonseca 2012) para caixas de madeira com dimensões internas de 15 x 15 x 6,5 cm. No interior de cada minicolônia, foram colocados dois alimentadores, um para a oferta de pólen e o outro para o xarope (1:1; água e açúcar). De acordo com as normas de biossegurança do CTNBio (Comitê Técnico Nacional de Biossegurança), as minicolônias foram mantidas sob restrição de

voo, uma vez que estas foram submetidas à alimentação com pólen de eucalipto GM. Para tanto, cobriu-se a entrada da colônia com tela de metal (1 mm) e tecido de voal, impedindo que as abelhas mantivessem contato com o meio externo e impossibilitando a entrada de insetos inimigos, a exemplo de forídeos (Diptera: *Pseudohypocera* spp.). As minicolônias foram mantidas em sala climatizada (28 ± 2°C, 70% U.R.) onde os experimentos foram realizados sob iluminação vermelha.

Configuração dos hortos de *Eucalyptus*

As amostras de pólen administradas às abelhas foram obtidas em dois hortos florestais do híbrido *Eucalyptus grandis* x *E. urophylla*, pertencentes a empresa FuturaGene Ltda. O horto experimental, localizado em Angatuba (23°29'28,20'' S, 48°35'59,4'' O), possui uma área de 1,3 ha com eucaliptos GM. As folhas de *E. grandis x E. urophylla* foram geneticamente transformadas via *Agrobacterium tumefaciens*, método descrito por González e colaboradores (2002), e cerca de 1.150 clones de plântulas GM foram randomicamente plantadas em 2007. Além destas, 50 plântulas não-GM (NGM) de mesma isolínea e idade foram igualmente plantadas, representando 4,2% das árvores. Como zona tampão, o horto experimental foi circundado por 300 m de campo. O horto comercial, com plantações de eucalipto convencional, de mesma isolínea, possui uma área de 600 ha, localizado em Alambari (23°31'44,83'' S, 47°50'53,72'' O), a 85 km distantes do horto experimental. Ambos os municípios pertencem ao estado de São Paulo, sudeste do Brasil.

Obtenção das amostras de pólen

As amostras de pólen foram coletadas de seis colmeias (modelo Langstroth) de *A. mellifera*. As colmeias foram dispostas nos hortos de eucalipto em janeiro de 2012, em pleno período de floração tanto das árvores GM como das árvores NGM. Três colmeias de *A. mellifera* foram posicionadas no centro do horto experimental, distantes 3 m entre si. As outras três colmeias foram similarmente instaladas no horto comercial, a 85 km do horto experimental, excedendo por completo o raio de voo das abelhas, o qual pode ser cerca de 10 km (Visscher e Seeley 1982). Em cada colmeia, acoplou-se um coletor de pólen, do qual, ao final de cada dia as amostras de pólen foram transferidas para tubos tipo eppendorf com capacidade para 1,5 ml e mantidas em freezer, a -20°C até o momento dos experimentos em laboratório. Durante este período, observou-se que abelhas coletavam ativamente recursos nas flores de eucalipto.

Quantificação do pólen GM nas amostras de pólen de eucalipto

A quantificação do pólen GM nas amostras foi baseada na técnica de *Real Time PCR (qPCR*), realizada pelo Laboratório EcoCerta (ISSO/ IEC 17025/ 2005). A fim de determinar a proporção de pólen de eucalipto GM nas amostras coletadas no horto experimental, utilizou-se um cálculo comumente empregado para avaliar de forma quantitativa outros transgênicos como soja, milho, algodão e canola:

$$\frac{\text{DNA (ng) amplificado do gene alvo (NPTII)} \times 100}{\text{DNA (ng) amplificado do gene de Referência (LFY1)}}$$

Desta forma, as amostras coletadas no horto experimental apresentavam 71% de pólen GM, enquanto que nas amostras coletadas no horto comercial, conforme o esperado, não detectou-se pólen GM.

Testes com minicolônias de *Scaptotrigona bipunctata* ***in vivo***

Antes do início dos experimentos, as abelhas já instaladas nas minicolônias, passaram por um período de dois a quatro dias de aclimatação à condição de confinamento. A fim de estimular o início da postura pela rainha, foi ofertado às minicolônias dieta proteica a base de pólen comercial multifloral de *A. mellifera* e xarope (1:1, água e açúcar), ambos *ad libitum*. Iniciada a postura dos ovos pela rainha, a alimentação com pólen multifloral foi interrompida para dar início aos experimentos com pólen de eucalipto. Eventualmente procedeu-se a retirada de acúmulos de resíduos das abelhas do interior das mincolônias.

As seis minicolônias foram submetidas a dois tratamentos: (1) dieta com pólen proveniente do horto convencional com eucalipto da mesma isolínea NGM (controle), e (2) dieta com pólen proveniente do horto experimental com eucalipto GM (teste). Os dois tratamentos foram administrados em sequência, em todas as minicolônias. Manteve-se um intervalo mínimo de dois dias com pólen comercial multifloral de *A. mellifera* antes do segundo tratamento. A administração de cada dieta deu-se por um período de 20 a 22 dias, até alcançar número mínimo de 10 novas células de cria/ minicolônia. A quantidade de pólen ofertada foi de 0,250 g/ minicolônia, em intervalos de 48h, em ambos tratamentos. Determinou-se esta quantidade de pólen, a partir de testes-piloto realizados previamente. Como fonte de carboidrato, foi oferecido xarope (1:1; açúcar e água) *ad libitum* durante todo o período de confinamento das abelhas.

Análises morfométricas e massa corporal de operárias em estágio de desenvolvimento pupal com pigmentação ocular

A partir de cada minicolônia, foram coletadas de 10 a 15 pupas de operárias/ tratamento (Tabela 1). Todas as pupas coletadas estavam em estágio de desenvolvimento pupal com pigmentação ocular. A massa corporal foi obtida com balança analítica de precisão (PUGDS, UY220), e, após, sob microscópio estereoscópio (OLYMPUS, SZ61) e paquímetro digital (MITUTOYO, IP66) mensuradas a largura da cabeça (LC) e a distância intertegular (DI) (Kuhn-Neto et al. 2009).

Tabela 1 Quantidade de pupas com pigmentação ocular de operárias de *Scaptotrigona bipunctata* coletadas/ minicolônia para realização das análises morfométricas e obtenção da massa corporal a partir da administração das dietas com pólen de eucalipto NGM e GM.

	Tratamento	
	Pólen NGM	**Pólen GM**
Minicolônia	n pupas	n pupas
1	15	15
2	15	10
3	12	10
4	12	12
5	12	12
6	15	15

Análises estatísticas

A fim de comparar as medidas morfométricas e massa corporal das pupas em cada minicolônia, a partir dos distintos tratamentos, utilizou-se ANOVA, OneWay. Para avaliar o grau de correlação entre os dados morfométricos obtidos e massa corporal das pupas, aplicou-se o teste de correlação de Pearson. Para ambos os testes utilizou-se o programa estatístico SPSS 17.0.

Resultados

Análises morfométricas e massa corporal das pupas de *S. bipunctata*

Comparando-se a massa corporal das pupas obtidas em cada minicolônia/ tratamento, observou-se semelhança (p = 0,166). Em média, as pupas apresentaram massa corpórea de 0,0212 g (SD = ±0,0028 g) quando alimentadas com pólen de eucalipto GM e 0,0204 g (SD = ±0,0019 g) quando alimentadas com pólen de eucalipto NGM (Fig. 1).

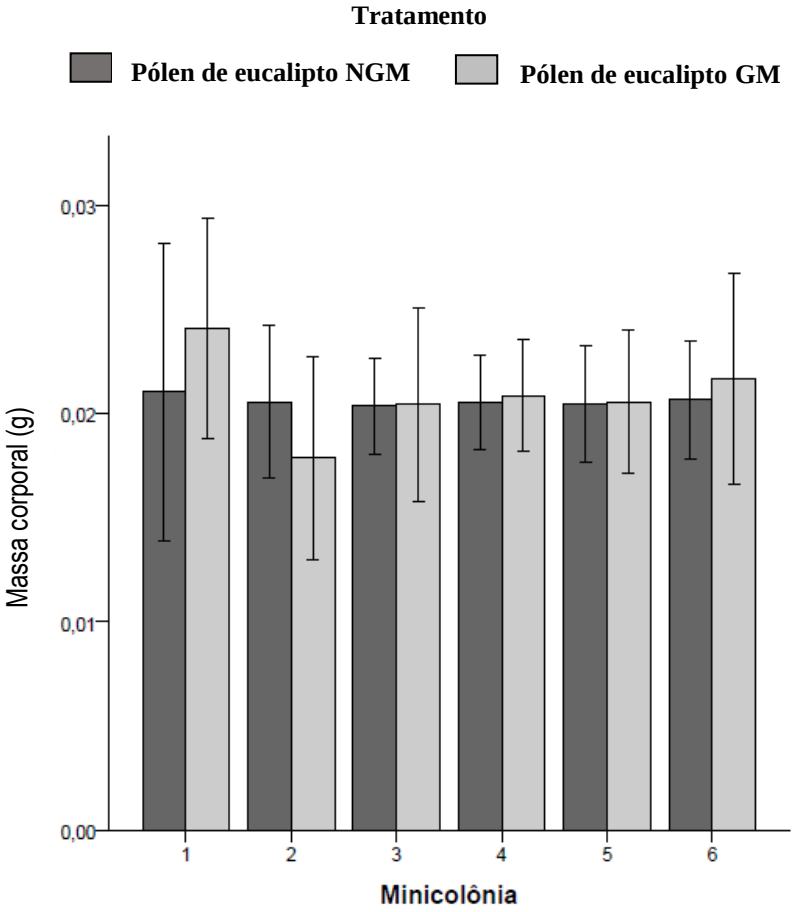

Fig. 1 Médias obtidas para a massa corporal de operárias de *Scaptotrigona bipunctata* em estágio de desenvolvimento pupal com pigmentação ocular submetidas a dietas com pólen de eucalipto NGM e GM (p = 0,166).

No que se refere às características morfometricas, diferenças foram observadas (p < 0,001), onde para a medida da largura da cabeça obteve-se em média 2,56 mm (SD = ±0,09 mm) no tratamento com

pólen GM e 2,51 mm (SD = ±0,06 mm) no tratamento com pólen NGM. Para distância intertegular, obteve-se em média 1,65 mm (SD = ±0,08 mm) no tratamento com pólen GM e 1,60 mm (SD = ±0,072 mm) no tratamento com pólen NGM (Fig. 2). O coeficiente de variação foi menor que 10% em todas as minicolônias, para ambos os parâmetros avaliados, em ambos os tratamentos. Os dados morfométricos apresentaram correlação positiva entre si e com a massa corporal das pupas para ambos os tratamentos (Tabela 2).

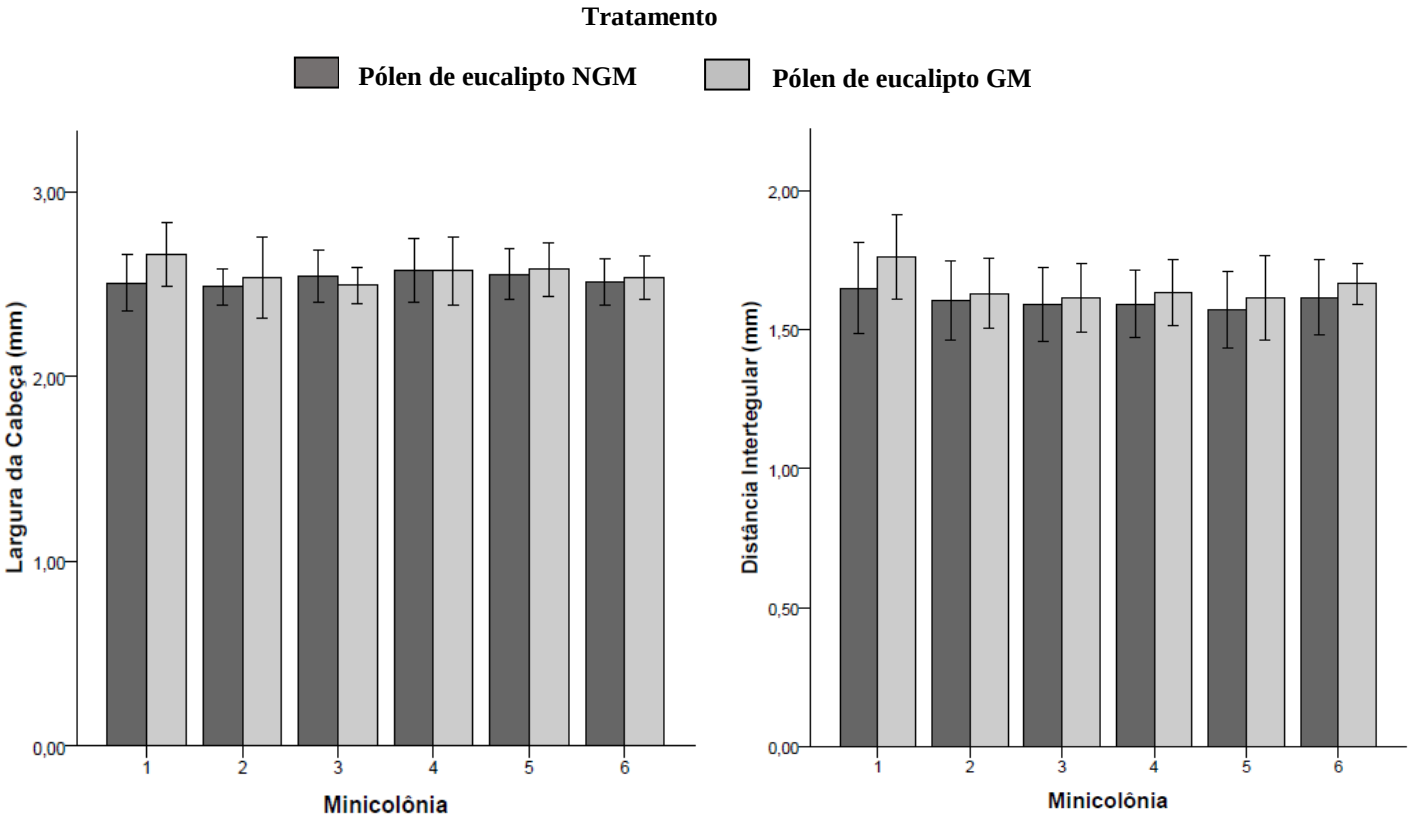

Fig. 2 Média dos valores obtidos para as características morfometricas de pupas de operárias de *Scaptotrigona bipunctata* ($p < 0,001$) submetidas a dietas com pólen de eucalipto NGM e GM. As barras sobrepostas indicam o desvio padrão.

Tabela 2 Correlação entre massa corporal e características morfometricas obtidas de pupas de operárias de *Scaptotrigona bipunctata* alimentadas com pólen de eucalipto NGM e GM ($p < 0,001$)

Tratamento com pólen NGM		
Medidas	**Massa Corporal**	**Distância Intertegular**
Largura da Cabeça	r = 0,307	r = 0,650
Distância Intertegular	r = 0,378	
Tratamento com pólen GM		
Medidas	**Massa Corporal**	**Distância Intertegular**
Largura da Cabeça	r = 0,519	r = 0,491
Distância Intertegular	r = 0,588	

Discussão

Apesar de exóticas, as árvores de *Eucalyptus* encontram-se amplamente distribuídas nos ecossistemas brasileiros (ABRAF 2013). Suas flores produzem expressivas quantidades de néctar e pólen, o que as tornam atrativas às abelhas e outros insetos (Marchini e Moreti 2003). Registros de Hilgert e colaboradores (2014) indicam que mesmo quando em pequena escala na paisagem, as abelhas coletam ativamente recursos alimentares em flores de eucalipto. Neste contexto, a forte atratividade das flores de eucalipto às abelhas traz a necessidade de testes de avaliação de risco de árvores GM de eucalipto a estes organismos não-alvo.

A tendência, no que diz respeito a testes de avaliação de risco de culturas GM a organismos não-alvo, é direcionada à culturas como o milho, soja e a canola, as quais expressam genes da bactéria *Bacillus turingiensis* para tolerância a herbicidas ou resistência a insetos (Hanley et al. 2003; Huang et al. 2004; Morandin e Winston 2005; Hendriksma et al. 2012; Hendriksma et al. 2013). No entanto, pouco se sabe sobre os efeitos dos transgenes implantados em cultivos arbóreos, a exemplo de *Eucalyptus* (Romeis et al. 2008; Wolt et al. 2010; Harfouche et al. 2011; Hilbeck et al. 2011; Romeis et al. 2011).

Efeitos negativos diretos de transgenes arbóreos expressos nas proteínas presentes no néctar e no pólen das flores de eucalipto GM não são esperados, pois os genes inseridos nas plantas são responsáveis pelo aumento da taxa de crescimento e qualidade da madeira, ou seja, sem caráter biocida. No entanto, de acordo com Theis e Lerdau (2003), não se pode descartar o fato de que efeitos secundários, provenientes de interação gênica, podem ocorrer, refletindo em alterações que poderiam comprometer a síntese ou degradação de metabólitos tais como alcaloides e terpenos. Os autores ainda reforçam que estes e outros metabólitos secundários possuem importante papel nas funções ecológicas, a exemplo de químicos defensivos produzidos pelas plantas. A alteração indireta do metabolismo da planta, determinado pela

expressão transgênica poderia, por exemplo, refletir em diferentes níveis de toxicidade ou alterações na composição nutricional do néctar e do pólen (Malone e Pham-Delègue 2001).

O pólen é fundamental para o desenvolvimento das abelhas e se constitui na principal fonte de proteínas (Nogueira-Neto 1970; Winston 1987). Em avaliações de risco de pólen e néctar, é importante considerar todas as fases de desenvolvimento das abelhas, uma vez que o contato com recursos florais se dá durante toda sua ontogenia (Winston 1987). Alguns autores consideram que a avaliação de toxicidade pode ser melhor evidenciada durante as fases imaturas, pois é quando as abelhas consomem maiores quantidades de pólen (Malone 2002; Brodsgaard et al. 2003; Hendriksma et al. 2012). No presente trabalho, a semelhança encontrada na massa corporal das pupas alimentadas com pólen de eucalipto GM e NGM, sugere que a composição dos pólens manteve-se semelhante, independente da modificação genética da planta.

A quantidade limitada de pólen destinado às abelhas durante os experimentos pode ter influenciado no comportamento de postura da rainha, uma vez que a produção de crias mostrou-se reduzida, em ambos os tratamentos, quando comparada a minicolônias com fornecimento de pólen *ad libitum*. Esta hipótese corrobora com experimento realizado com abelhas do gênero *Melipona*, no qual Roubik (1982) observou a diminuição da quantidade de cria produzida em períodos de seca, mesmo em colônias com estoques de alimento. Este achado sinaliza que o aprovisionamento das células de cria está relacionado ao fluxo de alimentos na colônia ou ainda que a redução da produção de crias pode ser decorrente da baixa qualidade do pólen coletado e estocado na colônia no período.

As diferenças morfométricas registradas nas pupas (largura da cabeça e distância intertegular) podem ser reflexo da variação genética dos indivíduos, uma vez que tratam-se de populações diferentes. Isto é reforçado pelo resultado obtido do coeficiente de variação, o qual foi menor que 10% para as pupas avaliadas, indicando grupos homogêneos.

O presente trabalho buscou verificar os potenciais efeitos da ingestão de pólen de eucalipto GM no desenvolvimento de operárias de *S. bipunctata*. Considerando os resultados da avaliação da massa corporal e morfometria das pupas descarta-se a hipótese de efeitos deletérios relacionados a ingestão do pólen de eucalipto GM sobre as abelhas

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CONCLUSÕES GERAIS

- O comportamento de postura de rainhas de *Apis mellifera,* bem como o sincronismo de desenvolvimento de imaturos em horto de eucalipto GM e NGM em floração foram similares.

- As diferenças morfometricas observadas em imaturos de *A. mellifera* expostos em hortos de eucalipto GM e NGM, não foram progressivamente relacionadas as distâncias.

- A massa corporal de imaturos de *S. bipunctata* tratados com pólens de eucalipto GM e NGM foi semelhante.

- As diferenças morfometricas observadas em pupas de *S. bipunctata* alimentadas com pólen de eucalipto GM e NGM podem ser reflexo da variação genética entre as distintas populações, uma vez que o coeficiente de variação indicou grupos homogêneos.

ANEXOS

NORMAS DE PUBLICAÇÃO

Capítulo I: Ecotoxicology and Enviromental Safety

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Capítulo II: Transgenic Research
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Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086

Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

Dissertation

Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

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www.issn.org/2-22661-LTWA-online.php

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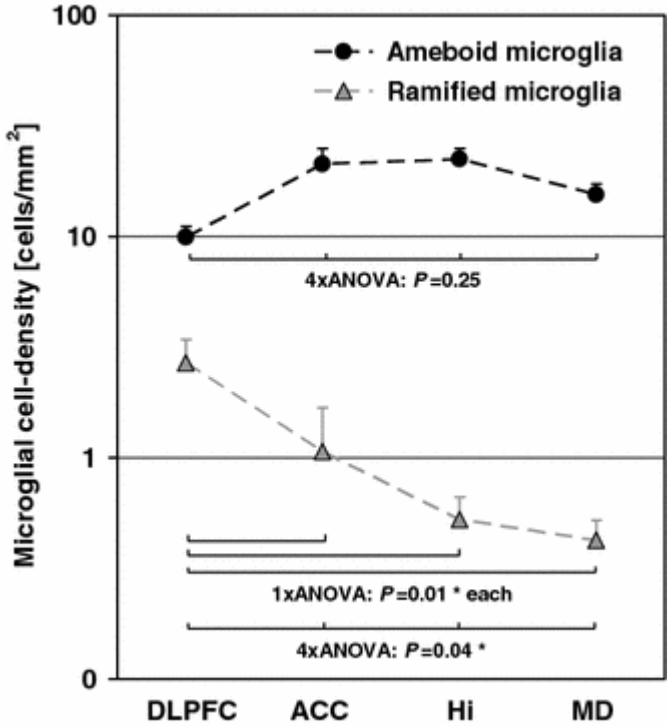

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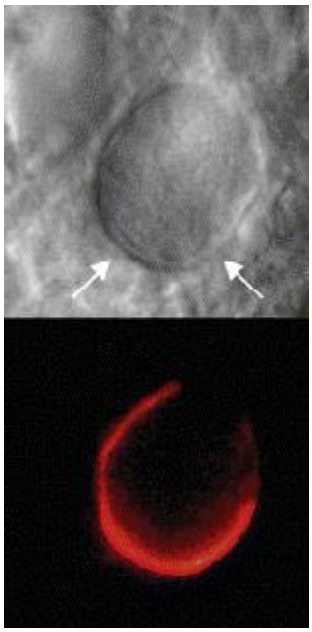

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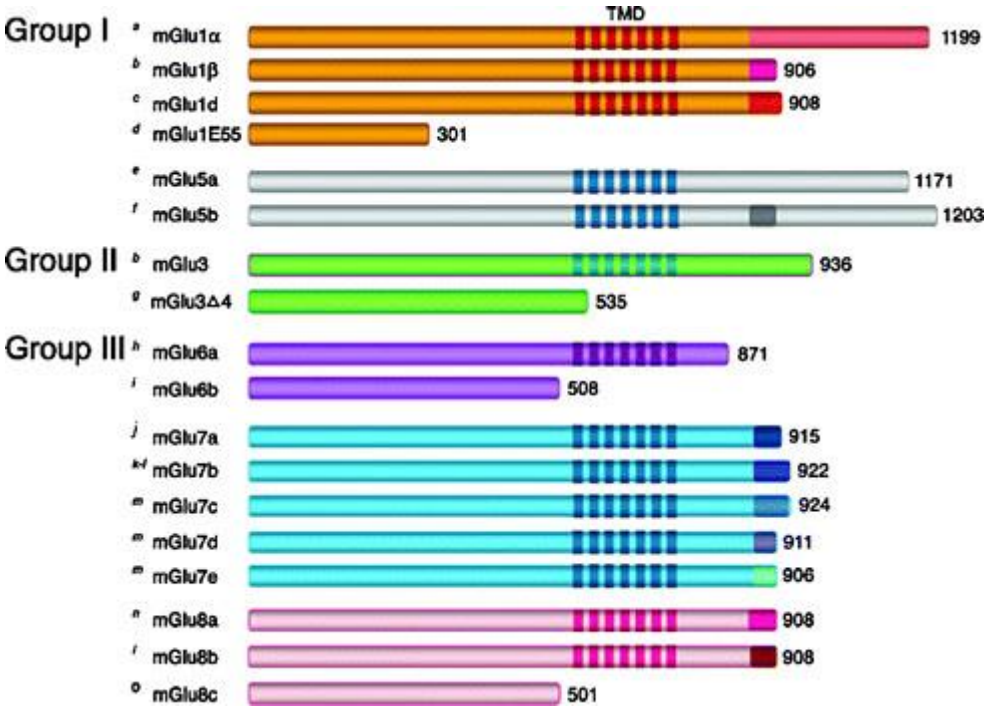

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