Three-dimensional reconstruction of *corpora allata* nucleus reveals insights into epigenetic mechanisms of caste differentiation in *Melipona scutellaris* stingless bees

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Abstract – In most Hymenoptera, the caste differentiation is influenced by differential environmental cues (e.g., food), which affect larvae development. However, *Melipona* stingless bees represent an exception to this rule. Workers and queens can emerge from brood cells of the same size, with the same quantity and quality of food. The different phenotypes of female bees depend on the interaction of genetic and environmental components. This mechanism is driven by the juvenile hormone, a key physiological factor synthesized by the *corpora allata* glands. In addition to that, epigenetic mechanisms were recently associated with caste development in *Melipona scutellaris*. In this study, using images captured by confocal microscopy and analyzed by 3D computational reconstruction, we show the similarities and differences in the pattern and volume of heterochromatin in the cell nuclei of *corpora allata* glands of *M. scutellaris*. Our results imply that heterochromatin territorial dispersion may act as a major player on the female phenotypic plasticity of *M. scutellaris* stingless bees.

DNA methylation / chromatin / YO-PRO-1-iodide / histone post-translational modifications / epigenetics

1. INTRODUCTION

In social insects, the females are divided into two castes: queens (fertile females) and workers

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(sterile or semi-sterile females) (Michener 2007; Cruz-Landim 2009). Different from bees of the genus *Apis* and in some stingless bees, in the genus *Melipona*, the developmental caste fate is not exclusively determined by diet (Campos 1979; Hartfelder and Engels 1998; Faustino et al. 2002). In these bees, diet seems to act as a limiting factor for queen development because morphologically identical larvae that had received a low quantity of food emerge as workers regardless of the genotype (Camargo et al. 1976). For larvae that had received adequate amounts of food, a genetic component is likely involved in

caste determination since a Mendelian segregation ratio of three workers for each queen has been observed in the offspring of well-fed colonies for many species (Kerr and Nielsen 1966; Kerr et al. 1975; Camargo et al. 1976; Maciel-Silva and Kerr 1991; Jarau et al. 2010). However, the molecular mechanisms related to these developmental routes remain largely unknown.

In *Melipona* bees, the exogenous treatment with juvenile hormone (JH), a sesquiterpenoid hormone synthesized by the endocrine glandules localized at the retrocerebral complex called *corpora allata*, is able to promote queen differentiation during the larval stage (Bonetti et al. 1995; Hartfelder and Engels 1998; Judice et al. 2004; Bonetti et al. 2006; Judice et al. 2006; Barchuk et al. 2007; Vieira et al. 2008; Siquieroli et al. 2009). Therefore, JH is responsible for the activation of caste-specific genes. Furthermore, it has been shown that the *corpora allata* glands of queens are bigger and have larger number of cells than workers of the same age (Kerr et al. 1975; Bonetti et al. 2006). These morphological differences are likely explained by the higher levels of JH in queens compared to workers (Cardoso et al. 2017a).

In addition to endocrine signals, gene expression can be altered through epigenetic factors acting on chromatin remodeling and consequently altering the accessibility of transcription factors to gene promoters (Allis and Jenuwein 2016). In honeybees (*Apis mellifera*), epigenetic factors, especially DNA methylation, can mimic the role of the royal jelly influencing the expression of caste-specific genes (Kucharski et al. 2008). The role of epigenetic mechanisms on morphologic and behavioral-associated traits has also been shown in ants (Alvarado et al. 2015; Simola et al. 2016) but remains largely unknown in stingless bees. Recently, epigenetic modifications were associated to caste development in *Melipona scutellaris* (Cardoso et al. 2017b), suggesting a potential link between epigenetic events and *corpora allata* activity.

To gain insights into the molecular regulation of phenotypic plasticity of *M. scutellaris*, we investigated whether the content and dispersion of heterochromatin in the nuclei of *corpora allata* cells (NCAC) can distinguish queens and

workers. Using computationally reconstructed images obtained by confocal microscopy, we have found a different heterochromatin pattern between castes. Our results indicate that the remodeling of the heterochromatin and epigenetic mechanisms may be involved in physiological processes associated with JH synthesis. Thus, this study fills an important gap in the understanding of reproductive division of labor of a non-conventional biological model.

2. MATERIAL AND METHODS

2.1. Bees

M. scutellaris bees were kept at the Meliponary of the Federal University of Uberlândia (UFU), Uberlândia, MG, Brazil (18°55' S/45° 17' W). Wax disks containing brood close to emergence were collected from colonies and incubated (31°C—75% relative humidity) until the newly emerged bees hatched. Three newly emerged queens (NEQ), two newly emerged workers (NEW) and one newly emerged queen produced after JH treatment (NEQ-JH) were used in this study. The nucleus volume, heterochromatin content, and its dispersion were assessed in 65 nuclei of NEW samples, 169 nuclei of NEQ samples, and 26 nuclei of NEQ-JH sample.

2.2. Treatment with juvenile hormone

Pre-defecating third larval instar of *M. scutellaris* ($n = 15$) were collected from the combs and transferred to petri dishes where they were topically treated with a single dose of 5 μ g of JH III (Sigma-Aldrich, St Louis, MO, USA) diluted in 1 μ l of acetone (Merck Millipore, Burlington, MA, USA). Control groups ($n = 15$) were composed by bees treated with acetone (Bonetti et al. 1995). The larvae were kept at 31°C with 75% relative humidity obtained with saturated KCl solution until adult hatching. Although it is not possible to distinguish morphological differences between queen-destined from worker-destined larvae in *Melipona* bees, we randomly sampled one individual from a petri dish of JH treatment in which 100% of the larvae remained alive and became queens. Thus, we ensured that

JH treatment was effective to induce queen-like morphological differences regardless of their genomic background.

2.3. Preparation of *corpora allata* of adults for confocal microscopy

Bees were anesthetized on ice and dissected in physiological solution for insects (128 mM NaCl, 18 mM CaCl₂, 1.3 mM KCl, 2.3 mM NaHCO₃) under stereoscope (Nikon). *Corpora allata* glands were stained with YO-PRO-1-iodide, a nuclear fluorescent dye that has been used to observe chromatin domains in fixed and live cells (Flors 2013). Dissected *corpora allata* glands were permeabilized with TRITON X-100 (0.1% v/v, Sigma-Aldrich) and sodium citrate (0.1% v/v) and held at room temperature for 1 h. Thereafter, the glands were washed with PBS three times for 5 min each, then incubated with 1 mM YO-PRO-1-iodide (Thermo Fisher Scientific, Waltham, MA, USA) for 45 min in a darkroom and finally assembled on a slide with glycerin and enamel-sealed. Images were obtained by confocal microscopy (FluoView 1000, Olympus, Shinjuku, TYO, Japan). Z-stacking was performed with a size of 0.414 μm for each optical slice.

2.4. Nuclei and heterochromatin volume evaluation

The methodology adopted to analyze and quantify the volume of the nuclei and heterochromatin in *M. scutellaris* cells is shown in Figure 1 and described elsewhere (de Morais et al. 2018). The following sections describe each step of the methodology.

2.5. Isotropic resampling, noise reduction, and nuclei segmentation

All confocal images were gathered into a 3D stack (3D volume), and regions outside the *corpora allata* were manually removed (Figure 1a). The volume was then resampled to become isotropic, as the original size of the acquisition voxel was not uniform (the slice thickness in the axial dimension (z -axis) resulted in a voxel size in this dimension that is 1.45–20 times higher than the

size in x and y dimensions). Trilinear interpolation was used in the resampling process, resulting in isotropic volumes with voxel size ranging from 0.25 to 0.4 μm depending on the sample (Figure 1b) (Schroeder et al. 2006; Costa and Cesar 2009).

Using a filtering process, the noise was reduced in the resampled volume (Figure 1b) when necessary. Two types of noise reduction were used accordingly to the type of the noise: a 3D Gaussian filtering ($\sigma = 1$) and a median filtering using a $3 \times 3 \times 3$ mask (Schroeder et al. 2006; Costa and Cesar 2009).

A process called segmentation was used to determine which voxels in the volume correspond to the nuclei (Figure 1c). A mixture of different techniques was applied to this step: (i) A simple thresholding (vortex intensity above 20) was used as first draft of the segmentation, which was enough to obtain several nuclei; (ii) an adaptation of 3D Watershed algorithm for those nuclei which were clustered was also used; and (iii) nuclei were split by using opening operation from mathematical morphology, i.e., several erosion operations using a $3 \times 3 \times 3$ cross-shaped mask followed by dilations using the same mask—the dilations were constrained to the voxels of the nuclei detected in the result of the first segmentation using thresholding. After segmentation, the nuclei were labeled using connected component labelling algorithm where nuclei with volume less than $44.7 \mu^3$ (empirically obtained) were discarded (Schroeder et al. 2006; Costa and Cesar 2009).

2.6. Heterochromatin region

Heterochromatin staining with single-molecule dyes, such as YO-PRO-1-iodide, produces images where the intensity of the voxels is proportional to the chromatin condensation (Simonson et al. 2011; Zessin et al. 2012; Flors 2013). That is the reason why the highest values correspond to the regions of heterochromatin inside the nucleus (Zessin et al. 2012). In order to identify the heterochromatin regions, a new threshold was applied to the resampled volume, but now individually for each nucleus (Figure 1c). The threshold value was chosen as the value corresponding to 40% above the mean intensity inside the nuclei.

Figure 1 Workflow with the methodology used to analyze and quantify the volume of nuclei and heterochromatin from *corpora allata* cells in *Melipona scutellaris*. **a** Original confocal image series. **b** 3D volume representation after gathering all images and after the application of algorithms for volume resampling and noise reduction. **c** Image segmentation process used for nuclei detection. **d** 3D reconstruction of all detected nuclei. **e** An example of a reconstructed nucleus, where it is possible to estimate its volume. **f** The same nucleus visualized with a transparent surface (green) and its heterochromatin (brown). Heterochromatin was also detected by using the image segmentation algorithm. At this stage, it is possible to estimate heterochromatin volume.

We used a percentage value above the mean to handle with possible variabilities in the fluorescent intensity of the cells. The same percentage was used in all images. During image acquisition, it may occur differences in the fluorescent intensity between different samples or even between different slices of the same sample, due to fluorescent attenuation along *z*-axis (depth axis). The value of 40% was chosen empirically, after testing values 20%, 40%, and 60% above the mean.

2.7. 3D visualization

After segmentation, the whole nuclear volume was reconstructed and visualized using marching cube algorithm, generating surfaces composed by

triangular meshes surrounding the nuclei and heterochromatin regions. The whole gland was reconstructed (Figure 1d), followed by the visualization of one of the analyzed nucleus (Figure 1e) and its heterochromatin region (Figure 1f, brown region) (Schroeder et al. 2006).

2.8. Quantitative and qualitative measurements

In order to estimate the volume of the nuclei (μm^3), the triangle mesh of the surface of the nuclei was used. The same approach was used for heterochromatin volume estimation (Schroeder et al. 2006). We also classified the cells into two groups according to the following

parameters: type I, “disperse” heterochromatin content was considered with one or more heterochromatin pixels unconnected, usually found in the center and peripheric regions of *corpora allata* nuclei; and type II, “centralized” heterochromatin content was considered the nuclei with centralized heterochromatin pixels and none splitted pixels. Only the most representative type of heterochromatin dispersion is shown in Figure 2.

2.9. In silico analysis of regional methylation status

To gain insights into the epigenetic regulatory mechanisms of genes coding enzymes of JH pathway, the DNA methylation levels were in silico predicted for two genes: farnesoic acid O-methyl transferase (*famet*) and methyl farnesoate epoxidase (*mfe*). In silico analysis was carried out using the software Emboss CpGplot (https://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/) and *M. scutellaris* gene sequence available at NCBI website (GenBank accession number: *famet*, AM493718.1; and *mfe*, KY768854.1). Based on the knowledge that methylated cytosines are hypermutable due to spontaneous deamination, leading to passive demethylation and CpG depletion over time, the observed to expected ratio (obs/exp) was calculated. This metric determines that regions with an obs/exp. ratio lower than one are predicted as methylated. As CpG islands are hypomethylated in mammals and social insects (Fouse et al. 2008; Meissner et al. 2008; Lyko et al. 2010), only regions outside CpG islands were identified as potentially methylated regions (supplementary Figure 1). CpG islands were defined as default by Emboss Cpgplot software (CG content = 50%). The obs/exp. approach has been successfully used to access epigenetic information in other social insects (Elango et al. 2009; Kucharski et al. 2015; Glastad et al. 2017).

2.10. Statistical analysis

All statistical analyses were performed by using GraphPad Prism software (Version 5.0).

Data were first $\log^{10}$ transformed to approximate to normal distribution, then tested with D’Agostino-Pearson omnibus test to verify homogeneity distribution of residuals. Differences in the nuclei size and heterochromatin volume between NEW, NEQ, and NEQ-JH were analyzed by ANOVA or Kruskal-Wallis tests, followed by Bonferroni or Dunns correction for multiple comparisons, accordingly to the homogeneity of residuals. Chi-square test was used to assess statistical differences between workers and queens in the number of cells with different heterochromatin patterns. A $p < 0.05$ was considered significant.

3. RESULTS

Queens and workers differ between their heterochromatin distribution inside NCAC (Figure 2). The majority of NCAC of NEQ show a concentrated heterochromatin at central position in the nucleus, whereas NEW bees present a disperse heterochromatin pattern along NCAC (Figures 2g–i and 3d). We verified that heterochromatin content of NEQ-JH (NEQ raised from JH-treatment) showed a pattern of heterochromatin dispersion similar to NEW (Figure 2h–i). The quantification of total volume (Figure 3a) and heterochromatin content (Figure 3b) demonstrated differences in the total volume between NEQ compared to both NEW and NEQ-JH (ANOVA followed by Bonferroni correction, $F_{(2,257)} = 6.36$, $p < 0.05$, Figure 3a). The total heterochromatin content of NCAC from NEQ and NEW individuals was significantly different (ANOVA followed by Bonferroni post hoc test, $F_{(2,257)} = 4.566$, $p < 0.05$, Figure 3b).

In order to verify whether the heterochromatin volume in NEQ samples reflected real differences in caste rather than a proportional increase due to nuclei expansion, we plotted the heterochromatin ratio in relation to total nuclei volume for queens and workers (Figure 3c). With this approach, we did not find any difference in the heterochromatin percentage of NEW and NEQ individuals (Mann-Whitney test, $U = 5417$, $p > 0.05$, Figure 3c), suggesting that the global heterochromatin volume itself is not related to caste-specific phenotypes.

Figure 2 Images of *corpora allata* cells nuclei of *Melipona scutellaris*. **a–c** Overall image of the *corpora allata* gland from a newly emerged workers (NEW), newly emerged queens induced by JH treatment (NEQ-JH) and newly emerged queens (NEQ). **d–f** Computational reconstruction of NEW, NEQ-JH, and NEQ nuclei. **g–i** Dispersion of heterochromatin (brown marks) in NEW, NEQ-JH, and NEQ.

In order to quantitatively evaluate the territorial differences among castes (Figure 3d), the heterochromatin dispersion was grouped into two categories: type I, disperse heterochromatin content; and type II, centralized heterochromatin content. We found a clearly distinct pattern between both castes (χ^2 test, $p < 0.0001$, Figure 3d) as previously reported in Figure 2g, i. Finally, we used in silico analysis to predict regions that are potentially methylated for two genes (*famet* and *mfe*) which encode critical enzymes involved in the regulation of the JH biosynthesis. We found two

potentially methylated regions flanking a large CpG island of the *famet* gene; however, the *mfe* gene was absent of CpG islands and presented five potential methylated regions (supplementary Figure 1).

4. DISCUSSION

Our data clearly show a distinct territorial dispersion of heterochromatin content and nuclear volume between queens and workers of *M. scutellaris* stingless bees. These results are in

Figure 3 Quantification of heterochromatin and nuclei volume in newly emerged bees of *Melipona scutellaris*. **a** Nuclei volume of newly emerged workers (NEW), newly emerged queens (NEQ), and newly emerged queens induced by JH treatment (NEQ-JH). **b** Total heterochromatin volume of NEW, NEQ-JH, and NEQ. **c** Percentage of heterochromatin volume in relation to nuclei volume of NEW and NEQ. Number of cells: NEW, $N = 65$; NEQ, $N = 169$; and NEQ-JH, $N = 26$. **d** Classification of heterochromatin dispersion in nuclei of *corpora allata* cells (sample size is shown inside graphs). The whisker boxes represent the 25th–75th percentiles, and dots represent cell samples above or below 1.5 times of intern quantiles; * $p < 0.05$.

line with a previous study (Bonetti et al. 2006) suggesting that the three-dimensional reconstruction of NCAC is a suitable approach to evaluate molecular differences between queens and workers of *M. scutellaris*.

Heterochromatin regions are important genomic domains that silence gene expression in eukaryotes (Rea et al. 2000). The heterochromatin is maintained through epigenetic modifications in histone tails and subsequent association of repressor multiprotein complexes (Wang et al. 2014). It has been shown that royal jelly components might facilitate caste switching through modulating histone post-translational modification levels in honeybees (Spannhoff et al. 2011; Wang et al. 2015). The recent mapping of honeybee chromatin structure found several regions with distinct histone post-translational modifications enrichment in

the head compartment, which contained *corpora allata* glands, of both queens and workers larvae (Wojciechowski et al. 2018). Interestingly, differences in the content of histone post-translational modifications were also shown to be caste-specific in *M. scutellaris* (Cardoso et al. 2017b). In addition, differences in heterochromatin dispersion have been previously suggested to regulate genomic silencing in *M. scutellaris* tissues (Mampumbu and Mello 2006). Therefore, our results suggest that heterochromatin domains and consequently gene silencing are important for the regulation of caste identity in *Melipona*.

In addition to epigenetic modifications occurring in histone residues, epigenetic modifications in DNA can also affect heterochromatin dispersion and consequently, impact gene expression (Fuks et al. 2000). It is known that DNA

methylation affects mammalian gene expression reducing the promotor affinity to transcription factors (Bergman and Cedar 2013). In a second stage, methylated CpG's sites can recruit repressor complexes to induce heterochromatin regions, ensuring their negative effect on gene expression (Fuks et al. 2000). In contrast to mammals, DNA methylation in social insects is restricted to gene-body regions and regulates gene expression and splicing events (Lyko et al. 2010; Li-Byarlay et al. 2013; Alvarado et al. 2015). Interestingly, dynamic fluctuations of global levels of DNA methylation were found throughout critical developmental steps of caste dimorphism in *Melipona* bees (Cardoso et al. 2017b) and also in other social insects, as honeybees and fire ant *Solenopsis invicta* (Lyko et al. 2010; Foret et al. 2012; Kay et al. 2016). Together, these findings suggest a potential cross-talk between DNA methylation and heterochromatin regions regulating the phenotypic plasticity in stingless bee.

JH has been used to explain the control of endocrine mechanisms associated with metamorphosis, reproduction, development of the gonads, determination of caste, behavior, polyphenism, and physiology in a large range of insects (Bonetti et al. 1995; Hartfelder and Engels 1998; Bonetti et al. 2006; Barchuk et al. 2007). Interestingly, the larval methylomes of *A. mellifera* show differences between queens and workers in genes related to JH synthesis pathway (Foret et al. 2012). It is attempting to speculate that *mfe*, a gene from JH pathway, is also regulated by epigenetic mechanisms. Some evidences support this potential regulatory link between epigenetic mechanisms and JH pathway in the context of caste differentiation: (i) *mfe* encodes the last enzyme of JH pathway, and its expression is restricted to *corpora allata* cells (Cardoso et al. 2017a); (ii) its expression is higher expressed in queens compared to workers (Cardoso et al. 2017a); (iii) its transcriptional profile correlates with *corpora allata* activity (Cardoso et al. 2017a) which has a different heterochromatin pattern between castes (this study); and (iv) potentially methylated regions were found in *mfe* genomic sequence (this study). Corroborating this hypothesis, we also found potential methylated regions in the *famet* gene, another gene from JH pathway that is differentially expressed and spliced in both castes of *M. scutellaris* (Vieira et al. 2008).

Forthcoming studies must uncover the epigenetic control of JH-related enzymes in order to enlighten the caste determination system in *Melipona* bees.

JH treatment had no direct effect on chromatin remodeling despite differences in adult phenotype. This curious result is in line with previous published data (Judice et al. 2006) which identified differences in the expression of caste-specific genes in NEQ and NEQ-JH of *M. quadrifasciata*. In addition, DNA methylation and JH hormone seem to be independent regulatory modules in young workers in honeybees (Cardoso et al. 2018). Although we focused on a developmental stage where the castes were already determined, this result suggests that JH is a downstream component of caste differentiation, rather than affecting heterochromatin dispersion on NCAC. Whether heterochromatin patterns of NCAC affect JH biosynthesis remains to be determined.

Our results shed light on developmental processes associated to caste differentiation of *Melipona* bees. More specifically, they indicate that gene silencing through heterochromatin patterns is a molecular mark of caste identity in *Melipona* and therefore that epigenetic mechanisms may be associated to physiological signals to regulate social traits in stingless bees.

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AUTHORS' CONTRIBUTION

BAM, UVC, and BME conceived and designed experiments. BNA, BLM, and FMB performed experiments. CJCAM, SHE, and TBAN analyzed the data. CJCAM, AGR, TBAN, and BAM wrote the paper. BAM and TBAN supervised the work. All authors read and approved the final manuscript.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest The authors declare that they have no conflict of interest.

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