10-hydroxy-2E-decenoic Acid (10HDA) is not a Caste-differentiation Factor in Melipona Scutellaris Stingless Bees

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10-hydroxy-2E-decenoic acid (10HDA) is not a caste-differentiation factor in *Melipona scutellaris* stingless bees

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ABSTRACT

In bees from genus *Melipona*, differential feeding is not enough to fully explain female polyphenism. In these bees, there is a hypothesis that in addition to the environmental component (food), a genetic component is also involved caste differentiation regulation. This mechanism has not yet been fully elucidated and may involve epigenetic and metabolic regulation. Here, we analysed the expression of the genes encoding histone deacetylases HDAC1 and HDAC4 and histone acetyltransferase KAT2A in *Melipona scutellaris*. We also investigated the metabolic profile of larvae and larval food to search for putative queen-fate inducing compounds. Finally, we assessed the effect of the histone deacetylase inhibitor 10-hydroxy-2E-decenoic acid (10HDA) - the major lipid component of royal jelly and hence a putative regulator of honeybee caste differentiation - on *Melipona* caste differentiation. The *hdac1*, *hdac4* and *kat2a* transcripts were expressed at all stages, with fluctuations in developmental stages and castes, which may be related to endocrine regulation. We did not identify the putative caste-differentiation factors, geraniol and 10HDA. Also, 10HDA was unable to promote differentiation in queens. Our results suggest that epigenetic and hormonal regulations act synergistically for drive caste differentiation in *Melipona* and that 10HDA is not a caste-differentiation factor in *Melipona scutellaris*.

Introduction

In the majority of social insects, female castes share the same genome; however, highly reproductive long-lived queens and facultatively sterile short-lived workers contrast in morphological, physiological, and behavioural traits. This fascinating biological phenomenon, in which one genotype produces more than one phenotype, is referred to as polyphenism [1–4]. It occurs in response to different stimuli: environmental (chemical, nutritional, physical, etc); physiological; or development related. In insects, generally, this process follows three steps: 1) initial perception of environmental stimuli; 2) neuroendocrine transmission of these stimuli to target tissues; 3) activation of epigenetics mechanisms that allows the setup of alternative transcription programs responsible for the establishment of distinct phenotypes [1].

The mechanisms that promote caste differentiation in stingless bees have not yet been completely elucidated. Differently from most Hymenoptera, in which this process is nutritionally driven, in stingless bee from the genus *Melipona*, an interaction between genetic and nutritional factors is proposed [5]. In *Melipona*, the supply of food in the brood cells is massive; queens, workers and males develop in undifferentiated brood cells, with the same size and the same quantity of food [5–7]. In addition, during larval development it is impossible to distinguish caste or sex, which makes studies on caste differentiation even more complex.

The model of genetic-feeding control for caste differentiation in *Melipona* was proposed by Kerr, in 1950, to explain the fact that under optimal colony conditions, up to 25% of total females develop in queens. According to this mechanism, the divergences between queens and workers would be determined

by the presence of two genes, with two alleles each. The double heterozygosis would lead to higher titers of juvenile hormone (JH), and, therefore, result in the differentiation of the larva into queen, provided that the amount of food ingested by the larva was sufficient. Homozygosis for one or both genes would lead to the development of workers, regardless of the amount of food received [5,6,8].

It is generally accepted that JH, an acyclic sesquiterpenoid hormone, is the main hormone that regulates caste differentiation and age polyethism in bees [9–11]. Topical application of JH in *Melipona's* third instar larvae promotes differentiation in to queens [9,12,13]. Additionally, a previous study suggested that the addition of geraniol, a compound of labial secretion of nurse bees, to larval food is another key factor for caste differentiation in *Melipona*. According to these authors, this compound can promote differentiation in queens, in larvae with genetic predisposition, reaching the percentage of 25% of queens predicted by the KERR hypothesis [14]. Our group has found evidence of olfactory function in *Melipona scutellaris* larvae [15], indicating that environmental stimuli, such as geraniol [14] or other larval food components, could be perceived by this olfactory system, triggering cellular responses involved in caste differentiation in these bees.

Currently, it is largely recognized that epigenetic mechanisms are associated with caste polyphenisms regulation [16–29]. Honeybees and bumblebees present different sets of microRNAs [30–35] as well as differences in methylation [16,21,36–41] according to the caste. Also, in *Apis mellifera*, histone post-translation modifications were described [42,43]. In this species, royal jelly is composed to up to 6% of 10-hydroxy-2E-decenoic acid (10HDA), a broad-spectrum histone deacetylase inhibitor (HDACi). 10HDA is potentially important

in the regulation of genes triggering queen differentiation [22,44]. The presence of epigenome-modifying compounds and microRNAs in larval food may indicate a role of nutrition in driving epigenetic patterns related to development and caste in these bees [24,44–46].

Concerning to stingless bees, works on its epigenetics remain limited. Recently, a complete set of genes involved in DNA methylation and in histone post-translation modifications in the genome of *Frieseomellita varia* was reported [47]. Moreover, in our previous studies, we demonstrated a functional methylation/demethylation system as well as post-translational modifications of histones in the stingless bee *Melipona scutellaris*. We found that both the levels of phosphorylation at threonine 3 of histone H3 (H3T3-P) and the levels of monomethylation at lysine 4 of histone H3 (H3K4-Me) are higher in newly emerged queens than in workers at the same age [48]. Analysing *corpora allata* glands, which are responsible for JH production, we have shown that the territorial dispersion of heterochromatin can be an important epigenetic mechanism associated with the phenotypic plasticity in castes of this species [49].

Histone acetylation is controlled by the balance of activities of two enzyme families: histone acetyltransferases (HATs) - which acetylate lysine residues of histones, promoting gene expression - and histone deacetylases (HDACs) - which remove these Ɛ-acetyl-lysine residues, promoting condensation of chromatin and reduction in gene expression [50–52]. In the current study, we investigate the importance of histone acetylation and nutritional factors to caste differentiation in *Melipona*. First, we characterized the mRNA levels of three genes related to the acetylation machinery during *Melipona scutellaris* development (*hdac1*, *hdac4* and *kat2a*). In addition, we examined the larval food

124 composition and the metabolic profile of *Melipona scutellaris* larvae to search for
125 putative queen-fate inducing compounds - such as geraniol and 10HDA. Finally,
126 we adapted the well-established protocol for JH treatment to evaluate the impacts
127 of topical application of 10HDA, as an additional strategy to investigate the role
128 of this molecule as a caste-differentiation factor in the stingless bees *Melipona*
129 *scutellaris*.

130

131 Results

132 Expression of histone acetyltransferase and histone
133 deacetylase genes are temporally related to JH titers in larvae

134 Quantitative real-time PCR was used to analyse the expression of two
135 HDACs encoding genes (*hdac1* and *hdac4*) and one HAT encoding gene (*kat2a*)
136 in the tree main critical developmental stages of *M. scutellaris* larvae (Figure 1).
137 The expression of both HDAC genes had a decrease in transcripts levels from L2
138 to L3.3 stages, which was significative only for *hdac1* (Dunn's multiple
139 comparisons test, P = 0.0051) but not for *hdac4*. The expression of both genes
140 were followed by an increase in LD stage. Interestingly, we noticed that this
141 expression pattern (high in L2 larvae, lower in L3.3 larvae and slightly higher in
142 LD larvae) was the same profile of JH titers [53]. The *kat2a* transcripts presented
143 an inverse expression pattern compared to HDAC genes and JH levels [53], which
144 rises from L2 to L3.3 (Dunn's multiple comparisons test, P = 0.042) and
145 decreases in the LD stage.

146

Histone deacetylases genes show caste-specific expression patterns

The expression of these three genes (*hdac1*, *hdac4* and *kat2a*) was also evaluated during *M. scutellaris* pupal development, in which morphological differences associated with caste and sex are distinguishable, and in newly emerged adults in both female castes (Figure 2). For *kat2a* transcripts, no significative difference between workers and queens was found (data not shown). Differentially, both HDAC genes had higher expression levels in queens than in workers at the first two pupae stages – Pw (Sidak's multiple comparisons test, $P < 0.0001$, for *hdac1* and *hdac4*) and Pp (Sidak's multiple comparisons test, $P < 0.0001$, for *hdac1* and $P = 0.0004$, for *hdac4*).

Metabolomic profiles of larvae bees

The fact that the epigenome is sensitive to the metabolic state [54–57] led us to investigate whether there are metabolic changes along the larval development of *M. scutellaris* related to epigenetic mechanisms and hormonal signalling, which may be associated with caste differentiation.

For the first time, to our knowledge, the metabolites present during the larval development of one stingless bee were identified and quantified by CG-MS. We identified 274 metabolites (Supplementary Table S1) with concentrations that vary between larval stages (L2, L3.3 and LD) and same stage samples. Among these 274 metabolites, 38 were found exclusively in L2, 66 in L3.3, and 15 in LD (Figure 3A).

Principal Component Analysis (PCA) was able to discriminate the samples from the three analysed larval stages, L2, L3.3 and LD, with stage L3.3 presenting

samples with more distinct metabolites profiles (Figure 3B). Likewise, correlation analysis indicates a very low correlation between the L3.3 samples (Figure 3C). Both the correlation analysis and the dendrogram show that individuals from L3.3 and LD stages presented a closer metabolic profile than between each of these stages and L2 larvae (Figure 3C and 3D).

The enrichment analysis indicates that for all analysed larval stages, there were significant enrichment for metabolites related to ammonia recycling and the urea cycle ($P < 0.05$). For L2 and L3.3 stages, there is also enrichment for galactose metabolism. Furthermore, L3.3 samples show enrichment for amino acids metabolisms, and LD for amino acids and purines metabolisms and for Warburg effect (Supplementary Figures S1-3).

Metabolites of Larval food

We identified 369 metabolites in *M. scutellaris* larval food (Supplementary Table S2), using GC-MS analysis. Neither geraniol nor 10HDA were identified in the larval food of *Melipona* bees. Only two metabolites (glycerol and 2,3-butanediol) were found in all samples (Supplementary Figure S4). There was significative enrichment for metabolites related to lactose degradation and galactose metabolism ($P < 0.05$) (Supplementary Figure S5).

10HDA-treatment induces mortality and does not promote caste differentiation

M. scutellaris larvae in stages L3.3 and LPD (third larval instar) were topically treated with 10HDA, a broad-spectrum inhibitor of class I and II HDACs, at 1.88 mM; 5 mM; 15 mM; 30 mM and 107 mM. None of the five concentrations

tested was able to induce differentiation of the larvae in queens and the highest concentration (107 mM) caused high mortality (62.77%) (Figure 4).

Discussion

Here, we verified the expression of *hdac1*, *hdac4* and *kat2a* transcripts in larvae, pupae, and newly emerged adults of *M. scutellaris*, and the fluctuations in the expression levels between developmental stages and castes reinforce the hypothesis of the subtle regulation of the caste differentiation process. Interestingly, in our metabolomics analysis, we found high intergroup variability in L3.3 samples and were not able to identify the putative queen-fate inducing compounds – geraniol for *Melipona beecheii* [14] and 10HDA for *Apis mellifera* [22,44].

Expression of HDAC encoding genes have not been studied during development in bees, however they were extensively investigated in beetles [58–60]. We detected higher levels of *hdac1* transcripts in the beginning of larval development, followed by a decrease in the intermediate stage, and by a subsequent increase in the last larval stage. Similar profiles of mRNA relative expression levels of *hdac1* had been previously described in *Tribolium castaneum*, in which HDAC1 expression is essential to larval survival [58,60]. In this beetle species, this gene presents higher transcripts levels in eggs and 1-day-old larvae and lower levels during larval development. The expression starts to increase in the late-larvae stages and reach a dramatic increase in 1-day-old pupae [58], as we also observed for queen but not for worker pupaes in *M. scutellaris*. In addition, recently, it was suggested a role for HDAC1 in embryogenesis and in eclosion in pea aphids [61]. All together, these evidences

indicate that HDAC1 may be potentially involved in insects development across different species.

Extensive research demonstrates that many biological factors – such as pheromones, hormones and differentially expressed genes – are necessary to reach the regulatory complexity of caste differentiation [62]. Accordingly, we have shown that the expression of *hdac1* presents an opposite profile from *kat2a*, and these patterns are temporally related to JH titers in larvae [53]. This suggests that epigenetic and hormonal mechanisms may act synergistically underlying gene regulation in *M. scutellaris* larvae. However, in respect to JH regulation on HDAC genes, our findings differ from the results obtained for *T. castaneum*. In this beetle species, higher JH titers lead to a reduction in *hdac1* transcripts and supressed HDAC1 action [58,63]. Thus, it is possible that the relationship between histone acetylation and JH titers vary between insect species, if such causal relationship exists.

The higher expression of *hdac1* and *hdac4* transcripts in the early pupal stages of *M. scutellaris* queens may represent a layer of regulation of caste-specific transcriptional programs. If their expression patterns are coherently associated with their protein products, queens may suffer a remotion of Ɛ-acetyl-lysine residues from part of their histones, promoting local chromatin condensation and punctual and refined control of gene silencing. In agreement with our gene expression data, it was demonstrated in honeybees that aged-matched workers and queens exhibit distinct patterns of histone post-translations modifications [42,43], including caste-specific regions of intronic H3K27ac marks identified in the worker caste [43].

After we found differences in expression of HAT and HDACs genes, we decided to investigate whether there are metabolic changes along the larval development of *M. scutellaris* related to epigenetic mechanisms and hormonal signalling. We were able to cluster samples in their respective larval stage according to their metabolic profiles. Samples from stage L3.3 presented more intergroup variability than samples from stages L2 and LD. This can be explained by the fact that it is impossible to identify sex or caste in *Melipona* larvae, being feasible to assume that L3.3 samples are from different sex or caste. In addition, L3.3 is the developmental stage in which JH acts, promoting caste differentiation [13]. Thus, it is possible that differences in JH titers, may lead to different metabolites sets, if these samples really are from different sex or caste.

All larval stages were mainly enriched for metabolites related to carbohydrates and amino acids metabolisms. This is not surprisingly, once the larval food – the only nutrient source for these larvae – is a complex mixture of fermented pollen (protein), honey (carbohydrate) and hypopharyngeal glandular secretions [64,65]. In addition, the enrichment for amino acids and purine metabolisms in the end of the third larval instar can be associated with tissue remodelling process, which occurs in this stage to promote metamorphosis.

Despite some exclusive metabolites founded in each stage, alone, these molecules could not be linked to epigenetic mechanisms or hormonal signalling. Furthermore, among the 274 metabolites identified in GC-MS, no metabolites directly linked to geraniol and 10HDA metabolisms were identified. Jarau *et al.* [14] have shown that the addition of geraniol, a compound present in *M. beecheei* labial gland secretions, to larval food increases the number of larvae that develop in queens. Plus, 10HDA, the main fatty acid in royal jelly from *A. mellifera*, is a

molecule with a potential role in caste differentiation in this species [22,44]. Our larvae metabolomics data indicate, however, that geraniol and 10HDA may be not act as queen-inducing factors in *M. scutellaris.*

To confirm our hypothesis, since geraniol and 10HDA were described in larval food of other bee species and could be one of the compounds of glandular secretions of *M. scutellaris*, we decided to also analyse the metabolites present in *M. scutellaris* larval food. We identified 369 metabolites and, the function enrichment analysis indicated significant enrichment for metabolites related to lactose degradation and galactose metabolism ($P < 0.05$). These results are expected, since larval food is the only energy source from the embryonic phase until the hatching of the imago, and are in accordance with the results found in food analysis of *Melipona quadrifasciata* [64]. Moreover, enzymes involved in carbohydrate metabolism, including α-galactosidase and ß-galactosidase, have been formerly identified in *M. scutellaris* larval food [66].

Surprisingly, different larval food samples shared few metabolites, indicating a great diversity of identified compounds. The metabolites shared by more than two samples are related to carbohydrate metabolism. One of the two metabolites shared for all samples is 2,3-butanediol is produced by fermentation by a variety of microorganisms [67]. Stingless bees' larval food is rich in bacteria, yeasts, and fungi, which ferment the food and produce a wide range of secondary metabolites [68–70]. The diversity of compounds may be the result of different levels of metabolism by different microbiotas present in each brood cell.

Again, we were not able to identify metabolites directly linked to geraniol, 10HDA and JH metabolisms in *M. scutellaris* larval food. This finding reenforces our hypothesis that neither geraniol nor 10HDA, alone, are capable of inducing

queen phenotype in this species. In *Melipona compressipes*, it was demonstrated that 25% of larvae reared in mixed larval food (food collected from different combs, homogenised, and redistributed) differentiate in queens; proving that there is no role for differential feeding in caste differentiation in these bees [71]. Indeed, a complex mechanism such as female polyphenism caste differentiation should not rely on a single molecule present in larval food.

Finally, we evaluated the effects of 10HDA topical application in *M. scutellaris* larvae to confirm if this HDACi is not involved in caste polyphenism in these bees, and as an additional strategy to investigate the role of histone acetylation in this process. None of the 10HDA-treatments was effective in promoting the differentiation of the larvae in queens. Critical periods in development are represented by intervals in which an organism is especially sensitive to environmental stimuli. In insect caste differentiation, these periods indicate increased sensitivity to some regulatory hormones, such as JH [28]. Topical application of JH in *Melipona* larvae, in the temporal window that corresponds to the end of the third larval instar (stages from L3.3 to LPD), leads to the differentiation of the larvae in queens; the treatment before that period causes mortality and; after that window, does not promote differentiation [13]. In this study, we performed 10HDA-treatments in the same time interval in which *M. scutellaris* larvae are sensitive to JH application, however, they were not responsive to the HDACi treatment. Moreover, the highest 10HDA concentration tested induced high mortality (62.77%) on the treated larvae, and this compound was not found neither in *M. scutellaris* larvae nor in larval food, suggesting that there is no target for 10HDA in this species. Therefore, 10HDA is not a caste-differentiation factor in *M. scutellaris* stingless bees.

Epigenetic modifications possibly represent an association between genotype and environment in the development of divergent phenotypes in *Melipona* females [48]. Our expression data of genes related to acetylation machinery reenforce previous findings of our group [48,49] that caste differentiation in *M. scutellaris* may be accomplished through an association of epigenetic mechanisms and endocrine signalling. Moreover, we showed that despite histone acetylation seems to be involved in *M. scutellaris* polyphenism, 10HDA alone it is not able to promote queen differentiation in this stingless bee species.

Material and Methods

Bees and Larval Food

Larvae, pupae, adult bees, and larval food were collected from *Melipona scutellaris* colonies kept in the Meliponary of Federal University of Uberlândia, Uberlândia, MG, Brazil. The classification of developmental stages followed previously described parameters [53,72]. For analysis of metabolites and volatile compounds, samples were collected in the same day and from a single colony.

RNA extraction, cDNA synthesis and RT-qPCR assays

Total RNA was extracted using TRIzol (Invitrogen) following the manufacturer's recommendations. Each sample consisted of individual whole-body larvae, pupae or newly emerged bees. RNA was treated with 10U of DNAse I RNAse free (Promega), following the manufacturer's recommendations. RNA concentrations and quality were measured in spectrophotometer at 260 nm (Marca). From the DNase-treated RNA, 1 µg were used as template RNA to synthetize the first strand cDNA using Oligo dT (15) (Invitrogen) and M-MLV

Reverse Transcriptase enzyme (Promega) according to the manufacturer's protocol. Each sample was analysed in duplicate with a quantitative PCR assay (QuantiNova SYBR Green PCR Kit, Qiagen) to evaluate the transcript levels of two HDAC genes (*hdac1* and *hdac4*) and one HAT gene (*kat2a*) using 2.5 pmol of each specific primer. The following cycling conditions were used in the RT-qPCR assays: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Simultaneously, the ribossomal protein *rpl32* gene was accordingly analysed for sample normalization; the gene has previously been validated for use as endogenous control for stingless bees RT-qPCR assays [73]. Product specificity was validated for all samples by running a melting curve analysis after the last amplification step. Relative expression values were calculated using the $2^{-\Delta\Delta CT}$ equation [74]. Primer sequences for RT-qPCR reactions were as follows: *rpl32*-F: 5' CGTCATATGTTGCCAACTGGT 3', *rpl32*-R: 5' TTGAGCACGTTCAACAATGG 3'; *hdac1*-F: 5' GGTCTGTAGCTGCTGCTGTGA 3', *hdac1*-R: 5' GCATGATGTAAACCACCACCCT 3'; *hdac4*-F: 5' AAGAATGCGTTCGGGCTTG 3', *hdac4*-R: 5' CGCGTTTTGGCAAGGTATCC 3'; *kat2a*-F: 5' TTACGAAGGGGCAACACTGA 3', *kat2a*-R: 5' CTTCCGTATGACAGCCGTA 3'.

Analysis of Metabolites of Larvae and Larval Food

The metabolomic analysis of larvae was performed with larvae of the second (L2) and third larval instar (L3.3 and LD) in sample and technical triplicates. For the larval food metabolomics, brood cells containing eggs were uncapped, the eggs removed and the food from each cell was individually transferred to a 1.5 mL microtube. The analysis of the food was performed in

sample quintuplicate and technical triplicate. Metabolite extraction and derivatization, gas chromatography, mass spectrometry analysis and metabolomic data analysis were performed using the same conditions and spectrometer described by Venturini et al [75].

Treatment with 10-hydroxy-2E-decenoic acid

Pre-defecating (LPD) and L3.3 larvae of *Melipona scutellaris* were collected from combs and transferred to petri dishes where they were topically treated with a single dose of 1 µl of 10-hydroxy-2E-decenoic acid (10HDA, Cayaman Chemicals, CAS Number 14113-05-4) diluted in ethanol, in the following concentrations: 1,88 mM; 5 mM; 15 mM; 30 mM and 107 mM. Control groups were composed by larvae treated with 1 µl of ethanol (vehicle) and larvae that did not receive treatment. The larvae were kept at 29-30 °C with 75% relative humidity obtained with NaCl saturated solution until adult hatching [76]. The individuals were classified by caste when they reached the pupae phase of white eye and white body (Pw). The worker or queen phenotype was identified by means of morphological characteristics already defined in previous works [12].

Statistical Analysis

Statistical analysis of gene expression were performed on GraphPad Prism (Version 8.00). As the data presented an asymmetric distribution, Kruskal-Wallis test was performed with a post-hoc Dunn’s multiple comparisons test. The hypothesis of equality was rejected for $P < 0.05$. For comparisons between castes, Two-Way-ANOVA was performed with a post-hoc Sidak's multiple

comparisons test. The relative mRNA levels were presented as mean ± SEM (standard error of the mean).

Metabolomics analysis were performed on the Metaboanalyst software (Version 4.00). The concentrations of metabolites were first normalized by the mass (g) of each larva / sample. Then, the values were normalized by the mean and standard deviation of each group. In Correlation Heatmap, Pearson R were applied for distance measure; in Hierarchical Clustering Dendrogram, Euclidean distance was applied, and Ward was used as a clustering algorithm.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

L.D.F.B. Conception, design, development of the methodology, analysis and interpretation of data, writing and revision of the manuscript. L.L.B. Development of the methodology and revision of the manuscript. S.M.M. Development of the methodology. T.S.R. Development of the methodology. J.R.C.S. Development of the methodology. G.V. Development of the methodology. A.C.P. Development of the methodology. P.H.G.G. Development of the methodology. C.U.V. Conception, design, writing and revision of the manuscript, technical and material support. A.M.B. Conception, design, writing and revision of the manuscript, technical and material support.

Competing interests

The authors declare no competing interests.

Figures

Figure 1: Expression of HDACs and HAT genes in *Melipona scutellaris* larvae. Relative expression of *hdac1* (A), *hdac4* (B) and *kat2a* (C) quantified by RT-qPCR. L2 = larva of the second stage; L3.3 = larva of the third stage in the third instar; LD = defecating larva. The graphs show mean ± SEM (n ≥ 5). Statistical analysis: Kruskal-Wallis test with a post-hoc Dunn's multiple comparisons test, $P < 0.05$.

Figure 2: Expression of HDACs genes in *Melipona scutellaris* workers and queens. Relative expression of *hdac1* (A) and *hdac4* (B) quantified by RT-qPCR. Pw = pupa with white body and eyes; Pp = pupa with white body and pink eyes; Pb = pupa with white body and brown eyes; Pbl = pupa with light pigmented body and brown eyes; Pbd = pupa with dark pigmented body and brown eyes; NE = newly emerged adult. The graphs show mean ± SEM (n ≥ 3). Statistical analysis: 2Way-ANOVA with a post-hoc Sidak's multiple comparisons test, $P < 0.05$.

Figure 3: Metabolomic profiles of *Melipona scutellaris* larvae. Venn diagram of shared metabolites between larval stages (A), PCA clustering of samples (B), Correlation heatmap of samples (C) and Hierarchical Clustering Dendrogram of samples (D). L2 = larva of the second stage; L3.3 = larva of the third stage in the third instar; LD = defecating larva.

Figure 4: Effects of treatment of *Melipona scutellaris* on the third larval instar with 10HDA. Survival rate (A) and Distribution of females in castes, queen and worker (B).

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Figures

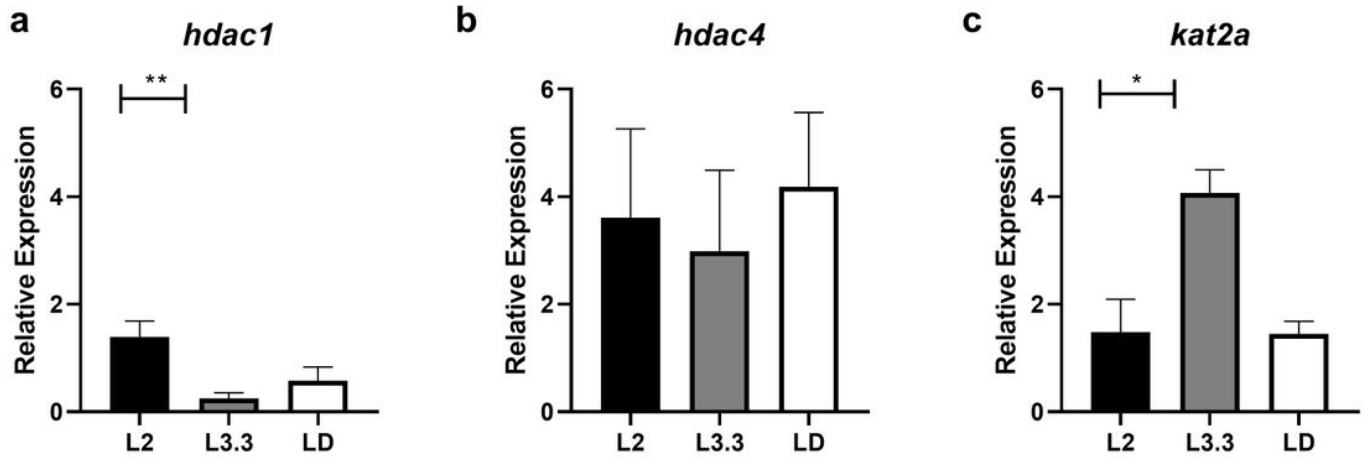

Figure 1

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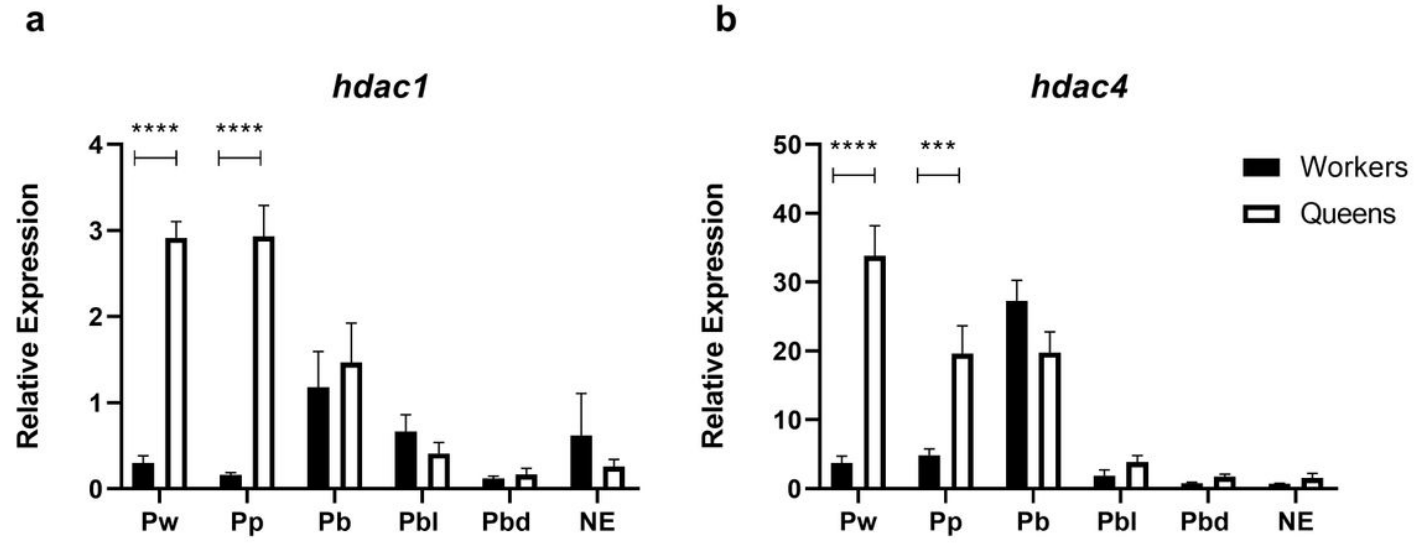

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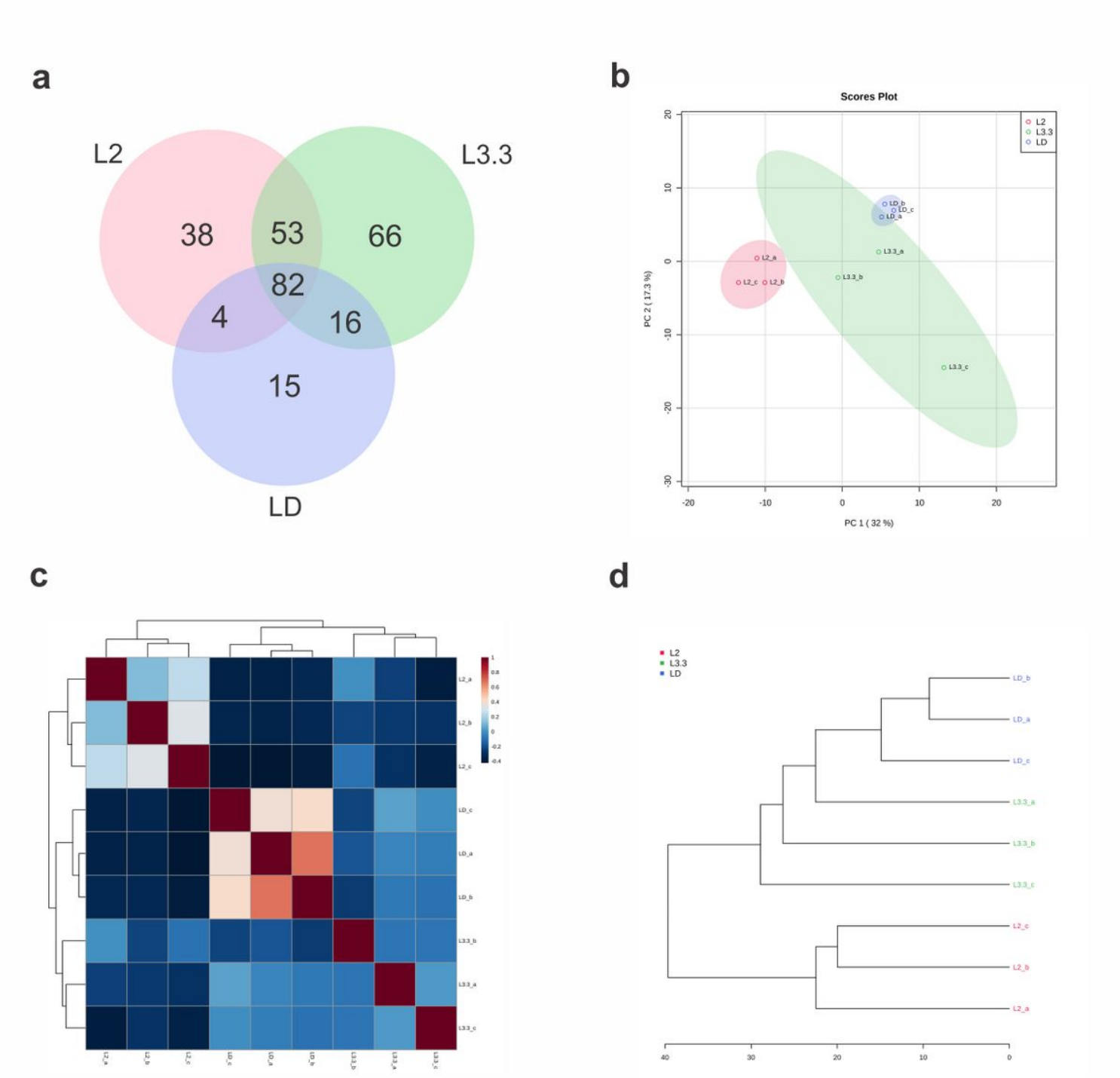

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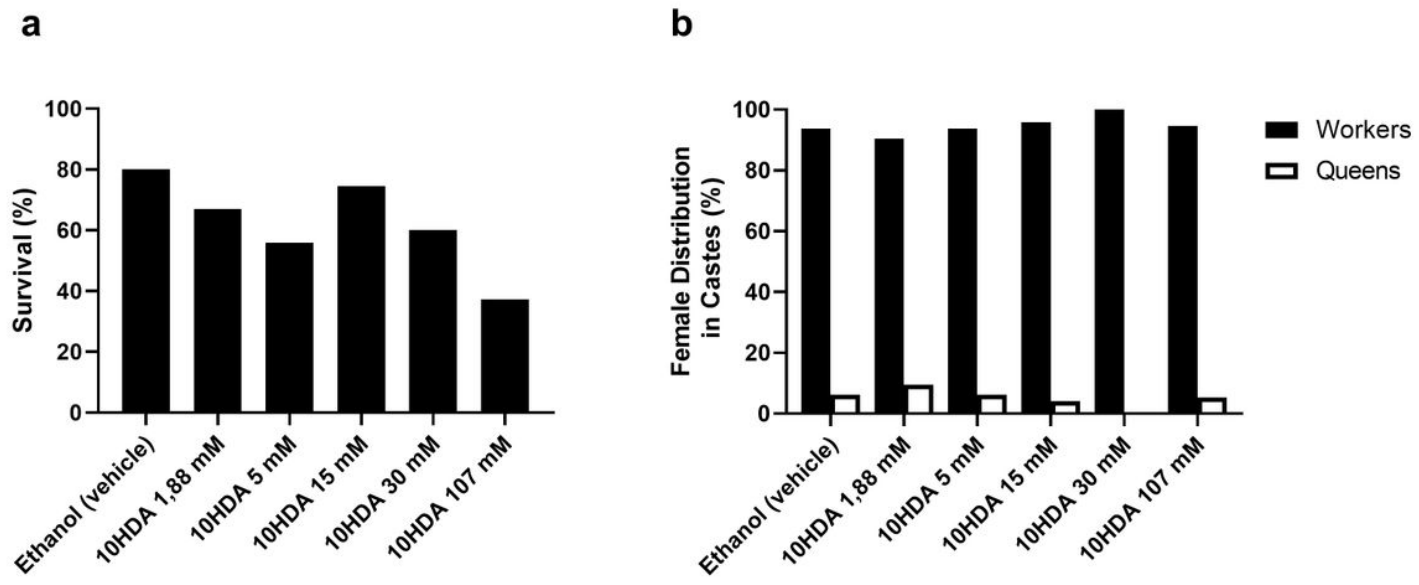

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