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ORIGINAL RESEARCH ARTICLE

Longitudinal survey reveals delayed effects of low gene expression on stingless bee colony health

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ABSTRACT

Bee populations are declining globally due to different environmental stressors, such as pathogens, malnutrition, and agrochemicals. Brazil is the home of hundreds of stingless bee species, some of them now considered endangered, though very little is known about the impact of disease on native bees. In Southern Brazil the endangered stingless bee *Melipona quadrifasciata* is affected by an annual syndrome that causes sudden death of workers, eventually leading colonies to collapse. Although novel viruses were found in foragers from diseased colonies, none has been consistently implicated in the outbreak. Here we conducted an integrative longitudinal survey on *M. quadrifasciata* managed colonies, measuring individual- and colony-level traits, to identify the causes behind the syndrome. We found that key genes related to xenobiotic metabolization, nutrition and immune responses are downregulated in foragers from colonies that became diseased two months later. The period that preceded the outbreak was also marked by pronounced forager weight loss as well as behavioural changes. Our findings reveal the syndrome may result from the stingless bee failure to respond to sublethal stressors and disruption of colony dynamics. These results support the proposition that worldwide bee mortality is influenced by a combination of diverse sublethal factors, and increase awareness of the long-term effects of genetic diversity erosion in stingless bee species, which can be enhancing their vulnerability to environmental stressors.

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Bee health; colony collapse; sublethal effects; colony dynamics; stingless bee; *Melipona quadrifasciata*

Introduction

The worldwide decrease in bee populations observed in the last decade is a matter of great concern. Some colony losses are explained by the presence of pathogens and other infectious agents (Evans & Schwarz, 2011; Schwarz et al., 2015) but several additional interacting stressors, such as habitat loss, malnutrition, agrochemicals and colony management practices are known to reduce the fitness of bee populations (Goulson et al., 2015). Theoretical studies indicate that although multifactorial stresses may cause colony failure, it is most likely a result of a critical level of stress from the accumulation of sublethal factors (Bryden et al., 2013).

One of the intrinsic properties of sublethal factors is their delayed effect on organismal fitness. In eusocial bees, where colony fitness is achieved by cooperation, sublethal factors characteristically compromise the ability of non-reproductive females to perform their regular tasks (Berenbaum & Liao, 2019). For example, honey bee workers reared in pollen-stressed colonies show normal preimaginal development, but as adults are less likely to waggle dance, and precisely inform food location (Scofield & Mattila, 2015). Exposure of larvae to sublethal doses of pesticides induces multiple changes in gene expression, leading to developmental and adult behavioural changes that weaken bee colonies (Berenbaum & Liao, 2019; Wu et al., 2017). Besides interacting with each other, environmental stressors are modulated by endogenous factors that are ultimately linked to the bee's genetic background (Poquet et al., 2016). Some bee management practices,

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including colony translocation, may lead to genetic homogenization, loss of genetic diversity, and the breakdown of local adaptations, finally impacting their capacity to respond to stressors (Espregueira Themudo et al., 2020; Jaffé et al., 2016).

The stingless bee *Melipona quadrifasciata* is one of the most extensively managed species and the object of intensive trading in Southern Brazil, where its nests virtually disappeared from nature (Fundação Zoobotânica, 2014). In this region, the practice of colony division has been performed for decades and became more common with trade intensification (Jaffé et al., 2015), which is now threatened by a syndrome that annually occurs in late summer, often leading to colony collapse (Caesar et al., 2019; Díaz et al., 2017). The first report of this syndrome was made by a beekeeper in 2014, which described it as a regular phenomenon that he observed since several years (Valmir Züge, personal communication). During the outbreak some worker bees from affected colonies show neurologic symptoms, such as tremors and paralysis, which are suggestive of poisoning or viral infections. In spite of having found novel viruses associated with symptomatic bees, such as dicistroviruses, which cause similar symptoms in *A. mellifera* (Genersch & Aubert, 2010), no virus (Caesar et al., 2019), and no other pathogen (Díaz et al., 2017) was found consistently associated with bees from diseased colonies.

Here we report an integrative and longitudinal study designed to uncover the causes underlying the annual syndrome of *M. quadrifasciata*. In addition to exploratory transcriptomic analyses, a temporal survey was conducted on three pairs of mother-daughter colonies kept in two separate localities, in order to evaluate the contribution of both genetic and environmental factors to the syndrome manifestation, by measuring individual- and colony-level traits.

Materials and methods

Searching for genes differentially expressed during the syndrome

Three *M. quadrifasciata* colonies were selected for exploratory transcriptomic analyses to identify differentially expressed genes during the syndrome outbreak. Two colonies were sampled during the outbreak in meliponaries from Bom Princípio (BP; 29°31′2.30′′S/51°17′29.00′′W) and Estância Velha (EV; 29°38′50.316′′S/51°10′23.592′′W). Both colonies were manifesting the syndrome signs, such as adult bee mortality, or bees presenting tremors and paralysis. A third colony, that showed no such signs, was sampled in BP. Three bees from each colony (labeled as D1, D2 and H, respectively) were collected and stored at −80 °C until RNA extraction for transcriptome sequencing. Total RNA of individual bees was extracted with TRIzol™ Reagent (Thermo Fisher Scientific, USA), following the manufacturer's recommendations. RNA yield was assessed with Qubit fluorometer (Invitrogen, USA) and the integrity was checked on a 1% agarose gel. For sequencing, aliquots of 2 μg RNA treated with TURBO DNase (Thermo Fisher Scientific, USA) from three foragers were pooled with respective colony samples. Transcripts were purified by polyA-tail selection, followed by library construction using TruSeq Stranded mRNA Library Prep Kit (Illumina, EUA). Single-end sequencing (read length = 150 nt) was performed on an Illumina NextSeq instrument, producing about 100 million reads per sample.

Trimmomatic v.0.36 was run with default parameters to remove low quality reads (Bolger et al., 2014). Gene expression (*GE*) was estimated with the *depth* command from Samtools v.1.3.1 (Li et al., 2009) by mapping the trimmed reads onto *M. quadrifasciata* genome (GenBank: GCA_001276565.1) with GSNAP v. 2018-07-04 (Wu et al., 2016) and recovering the number of reads per gene. To normalize gene expression (*NGE*) among the three transcriptomes the following formula was used: $NGE = (100{,}000{,}000/nr\ of\ mapped\ reads) * GE$. Next, the similarity in gene expression (*SGE*) between transcriptomes was estimated with custom Perl scripts by comparing *NGE* from healthy and diseased samples with the following formula: $SGE = (H + D)/(2 * max)$, where *H* is the *NGE* of the transcriptome obtained from the healthy colony, *D* is the *NGE* of the transcriptome obtained from a diseased colony, and *max* is the largest *NGE* value between *H* and *D* being compared. The result ranges between 0.5 and 1, and genes were regarded as differentially expressed (DEGs) when *SGE* was equal or lower than 0.7. Hypothetical genes among DEGs were re-annotated with BLASTp against the *nr* database (cutoff e-value 1e − 5) (Altschul et al., 1990).

To find functions over-represented in DEGs, a functional enrichment analysis was performed using the online version of g:Profiler with default parameters (g:SCS threshold of 0.05), and gene ontology annotation (GO terms) as data source (Raudvere et al., 2019). GO term similarity networks were performed with REVIGO, applying the medium (0.7) similarity threshold (Supek et al., 2011). The resulting interactive networks were used as input in Cytoscape v. 3.2.8 (Shannon et al., 2003) for further editing. DEGs with known roles for bee health were selected for relative quantification with RT-qPCR (see their putative biological roles on Table S1). Protein annotations of selected DEGs were cross-checked with the online version of eggNOG-mapper v2 (Huerta-Cepas et al., 2017), BLASTp analysis against *A. mellifera* proteins (taxid = 7460) and against the complete *nr* database of NCBI (cutoff e-value 1e − 5) (Altschul et al., 1990). Proteins were also compared to the Conserved Domains Database (CDD) using the Batch CD-Search tool (cutoff e-value 1e − 3) (Lu et al., 2020).

Monitoring bee colonies before, during and after the outbreak

In order to monitor changes of several key biological features, observations were made in *M. quadrifasciata* colonies under semi-controlled conditions during six months in two localities, i.e., Bom Principio (BP; 29°31′2.30′′S/51°17′29.00′′W) and Porto Alegre (PA; 30°2′4.7292′′S/51°13′3.5724′′W). The BP meliponary is located inside a small agricultural property where agrochemicals are regularly used. PA colonies were kept in the vicinity of a secondary forest located inside the University Campus. To control for genetic factors contributing to the syndrome, three colonies from BP (named BP1, BP2 and BP3) were divided in February 2018, resulting in three pairs of mother-daughter (MD) colonies. Daughter colonies (named PA1, PA2 and PA3) were translocated to PA after six months, when they became mature. All six colonies were monitored monthly from December 2018 (Summer) to May 2019 (Autumn). Each colony was equipped with a datalogger device (ONSET, Brazil) to record within-hive temperature and humidity. Records were made daily every six hours during the experiment, and downstream analyses were conducted with the daily lowest temperature (t) and the highest humidity (h). Additionally, a variable called delta (Δ = maximum value − minimum value) was calculated to evaluate the amount of daily variation in colony temperature (t) and humidity (h).

Pollen resources used by stingless bees

Aliquots of pollen stored by worker bees were collected with tweezers every month and stored in the laboratory at 4 °C. When no stored pollen was found within our experimental hives, or storage was inaccessible for sampling, pollen was sampled from non-experimental colonies located nearby. Pollen was chemically processed by acetolysis (Erdtman, 1952). Four slides were mounted for each sample with glycerin gelatine (Salgado-Labouriau, 2007) and around 500 pollen grains were identified at the family, genus or species level using reference material from the pollen library of the Palynology Laboratory at Universidade Luterana do Sul do Brasil (Ulbra), ICN Herbarium at the Universidade Federal do Rio Grande do Sul (UFRGS), database of the Pollen Catalogs Network (RCPol; www.rcpol.org.br) and pollen descriptions of plants from Southern Brazil (Bauermann et al., 2013; Evaldt et al., 2009; Liskoski et al., 2018; Radaeski et al., 2014).

Bee weight and relative quantification of gene expression

With an entomological aspirator, five foragers were collected monthly from each of the six experimental colonies, from December 2018 to April 2019. They were weighted in digital precision scale, and transferred individually to vials containing 200 µL of RNAlater (Thermo Fisher Scientific, USA), followed by storage at −80 °C for RNA extraction and gene expression analysis by RT-qPCR. Total RNA was extracted from the whole body and quantified as previously explained. An aliquot of 1 µg RNA from each forager was used as input for first strand cDNA synthesis with the High-Capacity Reverse Transcription kit (Thermo Fisher Scientific, USA).

Three DEGs were selected for monitoring forager bee expression patterns during the survey, namely putative mitochondrial *cytochrome P450* (*CYP450;* WN51_04136), *phenoloxidase* (*PO;* WN51_02761) and *apolipophorin* (*ApoLp*; WN51_14077). Primer3 from Geneious R11 (Kearse et al., 2012) was used to design primers based on each respective gene sequence (Table S2). Actin (*act*) and 40S ribosomal protein S5 (*rps5*) were used as references for gene expression normalization (Brito et al., 2015; Evans et al., 2006). StepOnePlus™ Real-Time PCR System (Applied Biosystems) was used for the RT-qPCR assays. Amplifications were carried out in 25 µL reaction solutions containing 12.5 µL cDNA (diluted to 1:30), 0.2 X SYBR™ Green I Nucleic Acid Gel Stain (Thermo Fisher Scientific, USA), 0.25 U of Platinum Taq DNA polymerase (Invitrogen, USA), 1 X PCR buffer (Tris-HCl 200 mM, pH 8,4, KCl 500 mM), 3 mM MgCl2, 0.1 mM of each dNTP, 0.2 µM of each specific primer.

Primer amplification efficiency was calculated with qBASE + software (Hellemans et al., 2007) from the slope of a five-point 1:10 serial dilution of calibrator cDNA samples (Table S2). Experimental setup of qPCR involved the sample maximization method (Hellemans et al., 2007), with three technical replicates for each sample, and inter-run calibrator samples were considered in calculations for run-to-run variation effects. The qBASE + pipeline was used for template quantification (Hellemans et al., 2007), by first calculating the means and standard deviations of quantification cycle (Cq) values of technical replicates, and relativizing Cq values based on the gene specific amplification efficiency. Next, the sample specific normalization factors were calculated by taking the geometric mean of the relative quantities of the two reference genes (*act* and *rps5*). The normalized Cq values were finally rescaled in relation to the sample with the lowest relative quantity (Hellemans et al., 2007), expressed in the form of calibrated normalized relative quantities (CNRQ), and used for statistical analyses. Detailed information regarding our RT-qPCR assays are provided as supplementary data (Table S2).

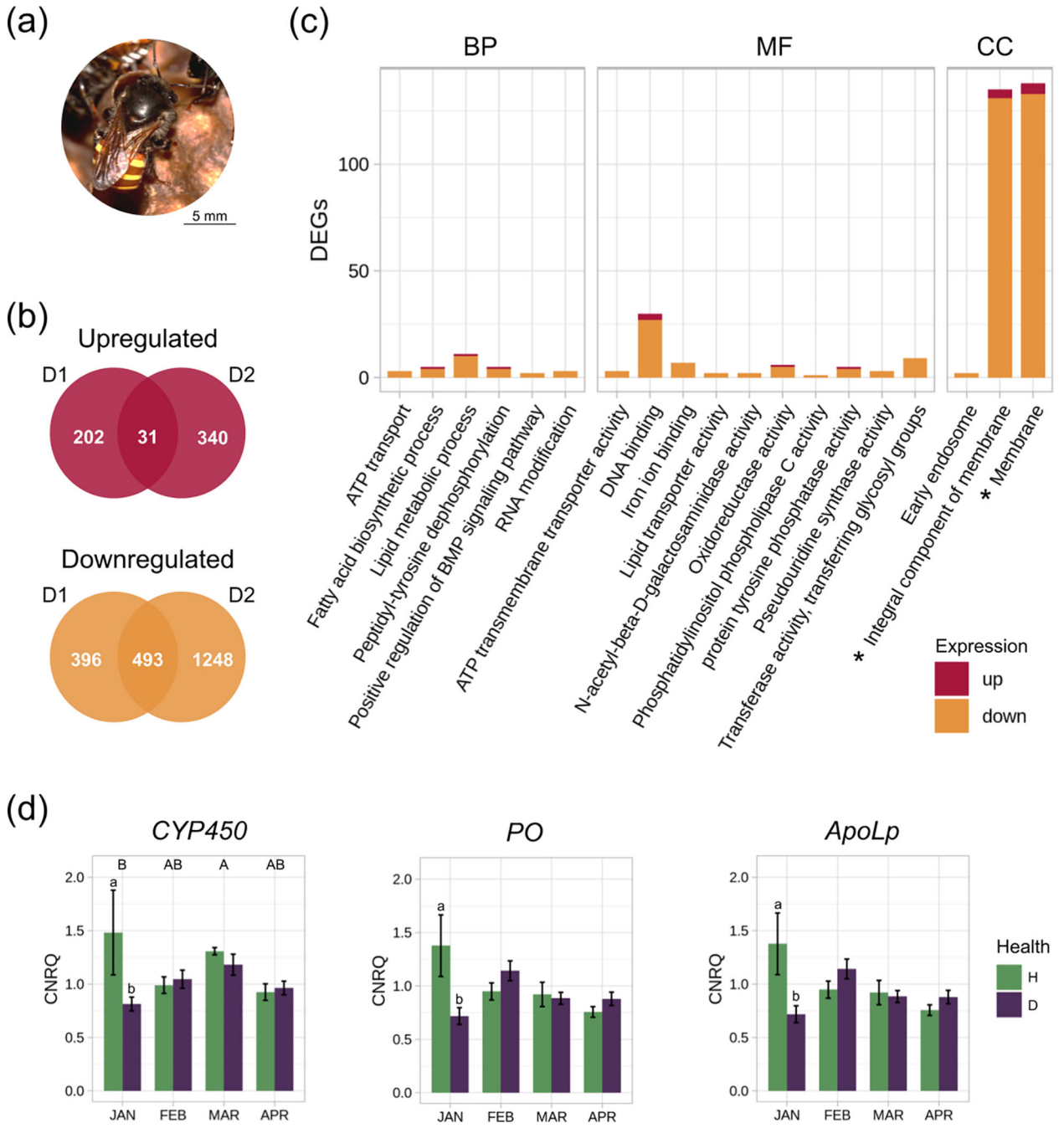

Figure 1. Worker bee gene expression patterns from transcriptome and RT-qPCR analyses. (a) *Melipona quadrifasciata* worker bee (Fototeca Cristiano Menezes, http://www.splink.org.br/search?lang=en&collectioncode=FCM). (b) Venn diagram showing differentially expressed genes (DEGs) up or downregulated in foragers from diseased colonies (D1 or D2). (c) Functional overview of DEGs based on gene ontology annotation ($P < 1$), with the asterisk indicating GO terms enriched in the DEGs ($P < 0.05$). Bars are sorted according to biological processes (BP), molecular function (MF) and cellular component (CC). (d) Barplot comparing relative expression (CNRQ) of *CYP450*, *PO* and *ApoLp* in foragers from colonies that remained healthy (H), with those that showed signs of disease (D) during the outbreak period. Bars indicate the standard error; uppercase letters indicate significant differences among months, and lowercase letters indicate significant differences between healthy and unhealthy colonies within months, according to Tukey's test ($P < 0.05$).

Statistical analyses

All analyses were conducted in R version 3.6.3 (R Core Team, 2019). Data were tested for their fit to normality and variance homogeneity using Shapiro's and Bartlett's tests ($P < 0.05$), respectively. When applicable, package MASS (Venables & Ripley, 2002) was used for Box-Cox transformation of non-normal and non-homogeneous data. Temporal differences in forager weight were assessed by one-way ANOVA using "month" as factor. Kruskal-Wallis test was used to assess the monthly differences of daily variations in colony temperature and humidity (Δt and Δh). To test whether unhealthy colonies were on average cooler and/or more humid, the March daily lowest temperatures and highest humidities were analysed by Mann-Whitney test. The package Laercio (Silva,

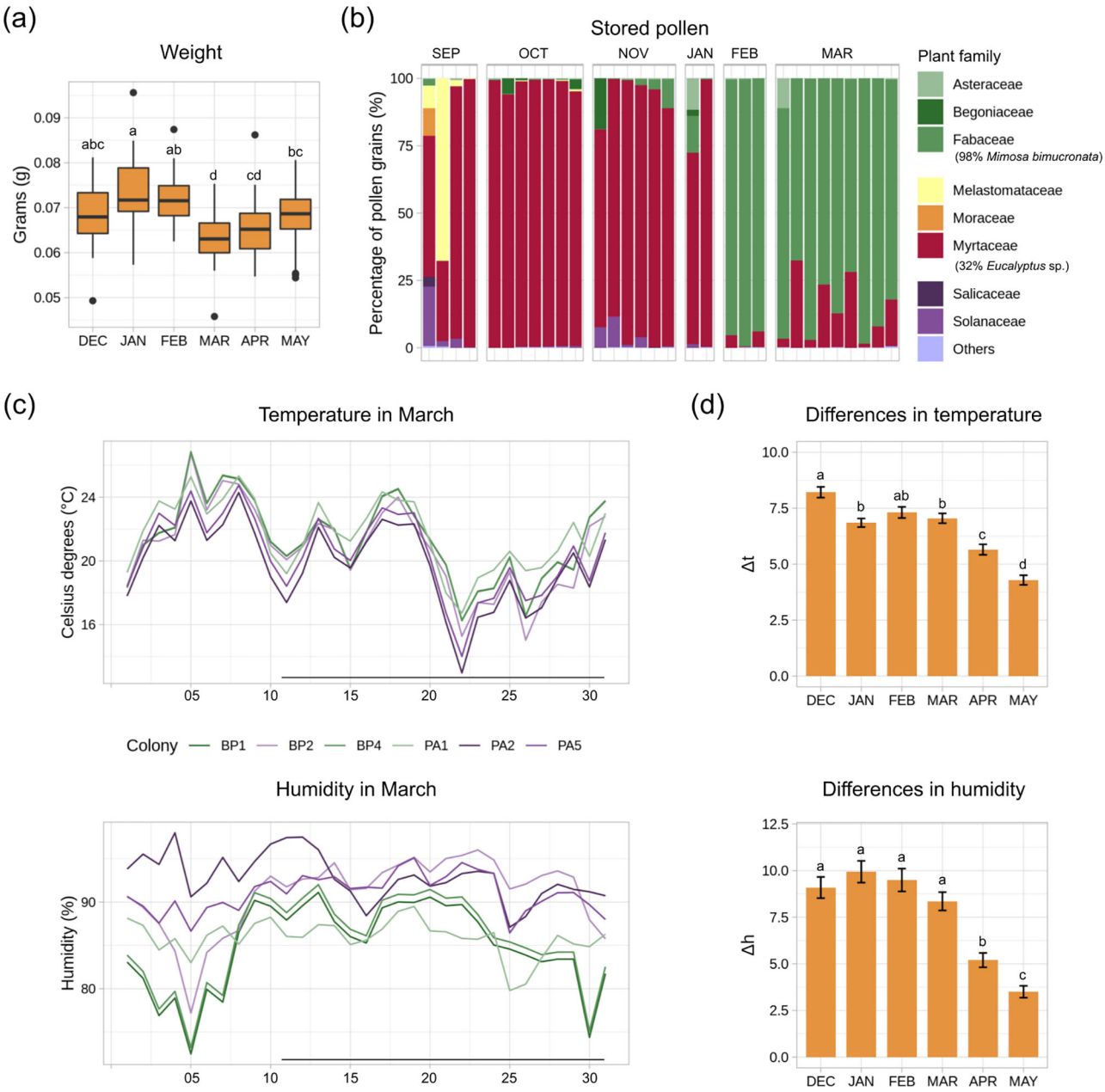

Figure 2. Changes in colony-level traits along the longitudinal survey. (a) Boxplot showing the monthly variation in average forager weight. (b) Barplot showing the monthly variation in stored pollen. (c) Line plot of the within colony daily minimum temperature and maximum humidity in March, as measured by a data logger. The colony health status during the outbreak period (shown by a horizontal line above the X-axis) is labelled by different colours (green = healthy; purple = diseased). (d) Barplot of the within colony average daily difference in maximum *vs*. minimum temperature (Δt) and humidity (Δh). Vertical bars indicate the standard error; lowercase letters represent significant differences according to (A) Tukey's test or (D) Nemenyi's test ($P < 0.05$).

2015) was used for mean comparisons with Tukey's test, and PMCMRplus (Pohlert, 2020) for Nemenyi's test. Forager CNRQ differences for *CYP450, PO and ApoLp* were evaluated by one-way ANOVA using "month" and "colony" as factors separately. Taking into account that the syndrome occurs in March, and that colonies have been monitored from January until April, a two-way ANOVA using either "colony", "MD colonies", "health status" or "intensity" combined with "month" as factors was also performed, enabling us to identify gene expression effects in specific periods during the course of our survey. Pearson's correlation coefficients among all traits were calculated with package Hmisc (Harrell et al., 2020).

Results

Differentially expressed genes during the outbreak

Transcriptome sequencing of D1, D2 and H foragers yielded 99,766,936, 102,034,731 and 135,982,124 single-end high quality-trimmed reads, respectively. From all reads, 87–99% mapped against the *M. quadrifasciata* genome. A total of 558 DEGs were

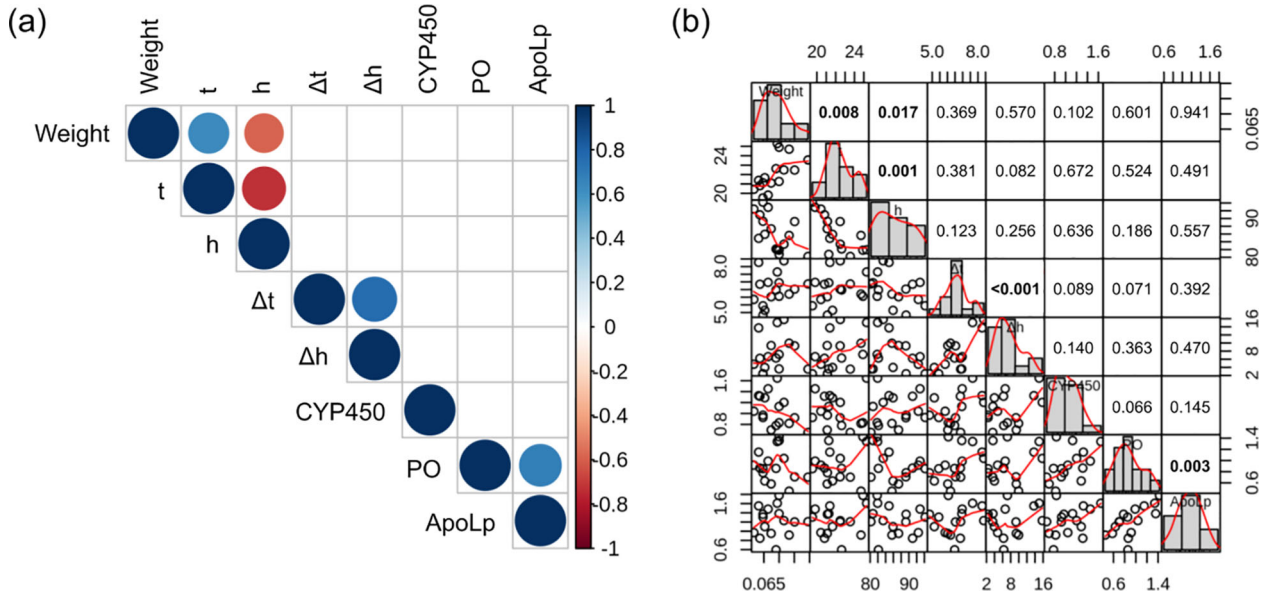

Figure 3. Pearson correlation matrix of all traits quantified at the colony- and individual-levels. (a) Significant positive and negative correlations are displayed in blue and red circles, respectively ($P < 0.05$). Colour intensity is proportional to the correlation coefficients (r). (b) Matrix with P values for the correlation coefficients (significant values in bold), histograms with kernel density estimation, and scatter plots with fitted line for each correlation.

found comparing bees from healthy and both diseased colonies, with 493 downregulated in foragers from diseased colonies (Figure 1B; Table S3). Membrane components (GO:0016020 and GO:0016020) are significantly enriched in DEGs (Figure 1C; Figure S1; Table S4), from which the vast majority is downregulated, representing major deficits in bees affected by the syndrome. Some GO terms, although not enriched, are relatively more frequent and highly connected to others, such as the biological process "RNA modification" (GO:0009451; Figure S2) and the molecular function "Transferase activity, transferring glycosyl groups" (GO:0016757; Figure S3).

To our knowledge, most of the highly differentially expressed genes were never directly implicated in bee health. Therefore, three DEGs homologous to genes with known role for bee health and commonly found differentially expressed in unhealthy bees from other species were chosen to quantify temporal variations in gene expression in foragers under semi-controlled conditions using RT-qPCR, i.e., *CYP450*, *PO* and *ApoLp* (Table S1). *CYP450* and *ApoLp* are both downregulated in our transcriptomes from diseased colonies, but curiously *PO* showed inconsistent patterns of differential expression in D1 and to D2 relative to H.

Outcomes of the survey

Among the six colonies monitored monthly in our study, four manifested signs of the syndrome in March 2019. Colonies BP2 and BP3 manifested the strongest signs of disease, with high mortality of workers, and some bees showing tremors or paralysis. Their respective daughter colonies PA2 and PA3 showed less intense signs and were characterized as mildly diseased. We rule out the possibility that bees died due to lethal doses of agrochemicals, since residue analyses conducted by NSF International Laboratories (Porto Alegre, Brazil) did not detect these compounds in three pools of ~20 bees from unhealthy colonies. The list of 197 compounds that have been tested, and their respective detection limits are available on Table S5. MD colony pair BP1 and PA1 did not manifest health alterations.

Temporal variation in gene expression

There was a significant temporal variation in the transcription of *CYP450* ($P = 0.009$), with peaks in March in both foragers from healthy and diseased colonies, and in January only in healthy ones (Figure 1D). Monthly variations in *CYP450, PO* and *ApoLp* gene expression showed similar interactions with the colony health status ($P = 0.0103$, 0.0152 and < 0.0005, respectively), and foragers from colonies that remained healthy during the outbreak period showed the highest expression of these three genes in January (Figure 1D).

Changes in worker bee nutrition and colony microclimate

We found a marked reduction in forager weight from January until March ($P < 0.0005$; Figure 2A), with the lowest average weight reached during the syndrome outbreak (March). From January to February we observed a sudden change in the pollen stored by worker bees, i.e., its composition

shifted from Myrtaceae (as *Eucalyptus* sp.) to Fabaceae (mainly *Mimosa bimucronata;* Figure 2B). Bee weight reduction was accompanied by a reduction in the internal temperature of colonies ($r = 0.63$, $P = 0.0088$), and increase in humidity ($r = -0.58$, $P = 0.0179$; Figure 3). This pattern was more pronounced in colonies that became diseased during the outbreak, i.e., MD colonies 2 (BP2 and PA2; $P < 0.0005$). Furthermore, temperature was lowest ($P < 0.0005$) and humidity was highest ($P < 0.0005$) inside diseased colonies when the syndrome symptoms were first observed in March (Figure 2C). The highest differences in daily temperature and humidity within colonies occurred from December to March ($P < 0.0005$; Figure 2D).

Discussion

Our study revealed that both genetic and environmental factors may influence the annual *M. quadrifasciata* syndrome. Mother-daughter colonies, exhibiting an average genetic relatedness of 0.375 (Oliveira et al., 2015), showed similar health status during the outbreak, despite having been kept in different localities. We suspect that the routine practice of colony division, associated with the concomitant loss of wild nests, may have reduced the genetic diversity of *M. quadrifasciata* populations, consistent with previous studies on managed colonies of this species in Southern Brazil (Koser et al., 2014). Reduced genetic diversity may have affected their ability to properly respond to environmental stresses in the form of sublethal factors such as agrochemicals, pathogens, and limited food resources, predisposing them to disease. We found that symptoms of worker bees were stronger in two affected colonies located nearby an agricultural setting. Thus, two lines of evidence allow us to suggest some of the mechanisms that can be involved in a higher susceptibility to the syndrome, and eventually colony failure, possibly through the impairment of forager responses to interacting stressors.

Firstly, our exploratory transcriptomic analyses showed that components of the cellular membrane, representing the layer that directly communicates with the environment, are enriched with DEGs that are downregulated in foragers from colonies affected by disease. Furthermore, enrichment analysis also indicated a slight over representation of lipid metabolic processes and oxidoreductase molecular functions, which were previously shown to respond to pesticides in honey bees (Wu et al., 2017). Secondly, foragers from our surveyed colonies affected by the syndrome in March expressed significantly less *CYP450, ApoLp and PO* in January. Bees rely on CYP450s for xenobiotic detoxification, which determines their sensitivity to agrochemicals (Manjon et al., 2018). ApoLp is responsible for lipid transport, being mostly involved in innate immunity (Kim & Jin, 2015). PO is an enzyme responsible for activating melanogenesis, an important defense mechanism of insects, and considered as an indicator of health condition strongly influenced by diet (González-Santoyo & Córdoba-Aguilar, 2012). Thus, it is not surprising that the expression patterns of these three genes (specially *ApoLp* and *PO*) are so remarkably similar in our survey, suggesting the combined involvement of three physiological processes in the *M. quadrifasciata* syndrome, i.e., xenobiotic detoxification, immunity and nutrition.

Although we were not able to detect agrochemical residues in the bees affected by the syndrome, we cannot rule out the contamination by sublethal doses months before the outbreak, such as in January. Agrochemicals are known to reduce ApoLp levels in honey bees (Nicodemo et al., 2018), and increase their demand for food (Balieira et al., 2018). Pollen stress in turn may also contribute to developmental and behavioural impairment (Scofield & Mattila, 2015). Moreover, January is characterized by a high density of pollinators in general, including *A. mellifera*, creating opportunities for getting in contact with a larger diversity of pathogens. *Melipona* spp. development takes around 40 days from egg to adult (Alves et al., 2009), thus *M. quadrifasciata* foragers sampled during the outbreak in March were immatures in January. Accordingly, we found a pronounced forager weight loss occurring between January and March, as well as a shift in the pollen stored by worker bees from Myrtaceae to mostly *Mimosa bimucronata* between January and February. Such a shift may result from the opportunity to forage *Mimosa,* that starts to bloom in February, but also from competitive exclusion, since *M. quadrifasciata* apparently competes with *A. mellifera* for Myrtaceae flowers (Wilms & Wiechers, 1997).

Interestingly, two months after *M. quadrifasciata* initiated storing *Mimosa,* a gradual recovery of forager weight was noticed. Colony recovery after the outbreak period is also suggested by a better performance of worker bees in controlling their nest microenvironment, as observed by the reduced differences in colony daily humidity and temperature in April and May. Nest and body thermoregulation are controlled by worker bees through specialized behaviors (Engels et al., 1995; Jones & Oldroyd, 2006) and appear to be important in creating a microenvironment for symbiont establishment (Hammer et al., 2020). Some gut bacteria seem to have a better performance at higher temperatures, and contribute to reducing pathogen infections (Palmer-Young et al., 2019). The effect of altered

thermoregulation on the maintenance of a healthy gut microbiota is still poorly understood in bees, and should be investigated in the context of this bee syndrome as well.

Unfortunately, it cannot be ruled out that the differences in forager bee gene expression observed in our study are due to age differences. Age polyethism is known to be a labile feature, since forager differentiation from nurses might be accelerated if the colony weakens (Huang & Robinson, 1999). Conducting invasive studies with stingless bees is not straightforward. Their colonies are much smaller than honey bees, ~300 workers (Dos-Santos et al., 2014), and have a different hive structure, which leads to a number of significant challenges for conducting experiments avoiding the side effects of excessive manipulation. For example, collecting 50 foragers from a *M. quadrifasciata* colony represents the loss of ~1/6 of its population, and significantly interferes on colony fitness. Furthermore, to access food stocks it is sometimes necessary to disorganize the hive construction by removing the nest involucrum or even the brood combs. Despite these challenges, our findings will guide future investigations, in perhaps more controlled experimental setups and with a larger number of colonies, by providing candidate bee health predictors and stressors involved in the *M. quadrifasciata* colony syndrome.

Although four of the six colonies surveyed in our study manifested some degree of the syndrome symptoms, none of them collapsed during the outbreak period. We think that the annual collapses reported for *M. quadrifasciata* colonies in Southern Brazil could result from positive density dependence influenced by the combination of diverse sublethal factors (Bryden et al., 2013; Khoury et al., 2011). By positive density dependence we mean that larger colonies are less likely to suffer from deaths to environmental stressors. Indeed, the PA colonies, which were only mildly affected by the syndrome, had also younger queens that normally produce more eggs, resulting in a larger population. The complexity of causes behind worldwide bee colony collapses demand efforts to sustain pollination services. Based on our findings, we suggest that practices such as limiting the use of agrochemicals in the vicinity of managed colonies and providing abundant natural polyfloral resources through the conservation of native forests, could help preventing the annual *M. quadrifasciata* syndrome. Furthermore, we recommend beekeepers to record annual mortality during the syndrome outbreak, and select for those stocks that better respond to the syndrome, i.e., colonies with fewer or no forager deaths. The above recommendations are useful for bee management in general, and rely on the premise that pollinator health cannot be sustained with a narrow deterministic view.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Ethics approval

According to Brazilian law, samples were collected with permission ICMBIO MMA 66382-2 and the access to the genetic patrimony by the A1B8F1F SisGen register.

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Data accessibility

Sequencing datasets are available at NCBI Sequence Read Archive (BioSamples: SAMN15728692, SAMN15728693 and SAMN15728694). Data and code for all analyses are available via GitHub (https://github.com/liliancaesar/Publication_scripts/tree/main/2020_Longitudinal_survey).

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