Investigating the molecular basis of cleptobiosis in eusocial stingless bees (Apidae: Hymenoptera)

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Abstract: Cleptobiosis is a widespread strategy among animals in which members of one species raid for food or other valuable resources that were obtained by another species. Obligate cleptobiotic species are however rare, and the eusocial stingless bee *Lestrimelitta limao* features amongst these rare species. This bee pillages other stingless bee colonies. To provide some insights into the molecular basis and evolution of cleptobiosis in eusocial bees, here we compare transcriptomic data of foraging workers of the robber bee *L. limao* and three non-robber stingless bees: *Nannotrigona testaceicornis*, *Scaptotrigona depilis*, and *Tetragonisca angustula*. Our findings demonstrate that most of the orthologs identified as differentially expressed (DE) among the robber and the non-robber bees are downregulated in *L. limao* workers, showing a reduced expression activity in the cleptobiont during foraging. Several DE orthologs potentially regulate neuronal and synaptic activities in addition to other critical cellular functions. Additionally, differentially expressed orthologs associated with floral resource exploration and energetic metabolism were identified. These genes warrant further investigation to enhance our understanding of the specific molecular adaptations associated with cleptobiosis in eusocial terms.

Keywords: *Lestrimelitta*; robber bees; Meliponini; Neotropical bees; parasitism

Introduction

Cleptobiosis is an ecological relationship in which an individual or a group of individuals from the same species loot food or other valuable resources from another (Breed et al. 2012). This behavior is widespread in vertebrates and invertebrates and can be facultative or obligatory, although obligate cleptobiosis is far less common (reviewed in Iyengar 2008). Often the term cleptobiosis is associated with species of social insects, especially ants and bees (Breed et al. 2012). It was first proposed as a classification for the ecological interactions of thief ants (Forel 1901; Wheeler 1910), where several cases of inter and intraspecific food theft are known (Hölldobler, 1986; Perfecto and Vandermeer, 1993; Yamaguchi, 1995; Richard et al., 2004; Ronque et al., 2018; Yamaguchi, 1995).

Among eusocial bees, facultative cleptobiosis is common in species from the family Apidae encompassing the tribes Meliponini (stingless bees) and Apini (honeybees), especially when species are facing food shortage (Bohart 1970; Wcislo 1987; Michener 2007; Costa et al. 2018). However, obligate cleptobiosis has only been described in two genera of stingless bees: *Cleptotrigona* and *Lestrimelitta*. Species of *Trichotrigona* are also likely to be obligate cleptobiotic based on their nest architecture, but no direct observations of this behavior have been recorded so far (Camargo and Pedro 2007; Pedro and Cordeiro 2015). The subgenus *Liotrigona* (*Cleptotrigona*) houses only one species – *Cleptotrigona cubiceps* – that is widely distributed across sub-Saharan Africa (Eardley 2004). In turn, the genus *Lestrimelitta* is comprised of 26 described species, all known to be cleptobiotic, and most of them (21 species) are of Neotropical occurrence (Marchi and Melo 2006; Camargo and Pedro 2013; Engel et al. 2023). The genus *Cleptotrigona* is closely related to the genus *Liotrigona* (Michener 2000), which belongs to the Afrotropical Meliponini clade, while *Lestrimelitta* belongs to the Neotropical Meliponini clade (Rasmussen and Cameron 2010). These two clades diverged more than 70 million years

ago (Rasmussen and Cameron 2010). Thus, obligatory cleptobiosis in bees arose independently from flower-collecting ancestors at least twice.

Lestrimelitta and *Cleptotrigona* species attack nests of other stingless bee species to rob pollen and nectar (Portugal-Araújo 1958; Wittmann et al. 1990; Sakagami et al. 1993; Rech et al. 2013). Accordingly, these species have morphological and behavioral adaptations likely driven by their cleptobiotic behavior. Maybe the most striking of these adaptations is the absence of a functional corbicula, a morphological feature common to all corbiculate bees (Sakagami and Laroca 1963b; Wille 1983; Eardley 2004; Michener 2007). The reduction or complete loss of pollen-collecting structures seems to be a convergent adaptation in parasitic bees, also observed in cleptoparasitic (brood parasites; *e.g.* Nomadinae bees) and social parasitic species (*e.g. Bombus Psithyrus* subgenus) (Michener 2000). Queens of *Lestrimelitta* have a higher number of ovarioles per ovary (10-15) than most stingless bees (~4) (Sakagami 1982), enabling the production of a larger number of workers. Moreover, the robust mandibles of the *Lestrimelitta* workers (Nogueira-Neto 1997; James et al. 2022) help them overcome the colony defenses of host species. An important behavioral adaptation of *Lestrimelitta* and *Cleptotrigona* species is their foraging strategy, they carry out organized robbing raids in which several individuals simultaneously attack colonies of other species; in *C. cubiceps* the number of individuals participating in these raids may reach a few dozen (Portugal-Araújo 1958), while in *Lestrimelitta* it can exceed several hundred (Sakagami et al. 1993). Raiding recruitment in *Lestrimelitta* has been reported to involve the use of semiochemicals (Blum 1966; Sakagami et al. 1993) that have toxin functions (James et al. 2022), and may also play a role in the host response to raids (von Zuben et al. 2016; James et al. 2022). Likewise, in an apparent strategy of chemical deception, it has been suggested that *Lestrimelitta* may

share cuticular hydrocarbons with their preferred host species to better conceal their presence (Vázquez et al. 2022).

Among all robber bees, *Lestrimelitta limao* is the best known and most studied species (Rech et al. 2013). They are named after the volatile citral compound produced and released by the workers, even though this compound is common to all species of *Lestrimelitta* (Blum et al. 1970; Francke et al. 2000). Like other species of *Lestrimelitta*, *L. limao* specialized in raiding stingless bee nests (Wittmann et al. 1990), although there are records of pillaging in honeybee colonies (Sakagami et al. 1993). There are records of *L. limao* raids in more than a dozen of stingless bee species, from different genera, including: *Friesella*, *Frieseomelitta*, *Melipona*, *Nannotrigona*, *Plebeia*, *Nannotrigona*, *Scaptotrigona*, *Tetragonisca* and *Trigonisca* (reviewed in Grüter et al. 2016). Nevertheless, this species appears to prefer certain hosts depending on the locations, and on the composition of the local stingless bee fauna (Sakagami and Laroca 1963b). *L. limao* raid attacks are common enough for this species to be considered a great threat to meliponiculture activity, as it attacks nests of species of commercial interest, killing many individuals, and sometimes, leading to the colony death (Sakagami and Laroca 1963b; Sakagami et al. 1993). In natural environments, it has been hypothesized that this species may control the size of stingless bee populations (Nogueira-Neto 1997). The cleptobiotic interactions between *L. limao* and other stingless bee species may have even driven the evolution of a soldier sub-caste specialized in colony defense in the assaulted species (Grüter et al. 2012, 2017). The number and size of these soldiers are affected by the colony exposure to cleptobionts (Segers et al. 2016), demonstrating the relevant role of cleptobiosis in driving the evolution of host species.

Despite the evolutionary, ecological, and economical relevance of cleptobiosis in stingless bees the mechanisms involved in the foraging strategy of *L. limao* and how they

diverge from other non-parasitic bees are poorly understood. Likewise, the adaptations at the molecular level related to the evolution and maintenance of obligatory cleptobiosis in eusocial bees are unknown. Herein, we investigated the molecular mechanisms involved in stingless bees cleptobiosis using comparative transcriptomics. We generated transcriptomic data from foragers of the robber bee *L. limao* and for three pollen-collecting (non-parasitic) stingless bees, *Nannotrigona testaceicornis*, *Scaptotrigona* aff. *depilis*, and *Tetragonisca angustula*. These data were comparatively analyzed in search of identifying and describing the molecular pathways associated with the cleptobiotic strategy of this robber bee.

Methods

Sample collection and RNA sequencing

Workers were collected while they were performing foraging activities, between 11:00 and 13:00h to control for clock gene differences. The workers of *L. limao* were collected from three natural nests in the city of São Paulo (Brazil); these workers were sampled when returning to their nests. Foragers of *N. testaceicornis* and *S.* aff. *depilis* were obtained from three colonies housed at the Bee Laboratory at the Universidade de São Paulo. For these species, workers were marked at their emergency and returned to their nests. After 30 days, period in which workers of several stingless bees are known to perform forager tasks outside the nest (see Grüter 2020), the marked bees were collected. For *T. angustula*, we used RNA-Seq data generated in a previous study from foragers sampled in a similar manner (Araujo and Arias 2021) and, to treat our data for possible sequencing batch effects, we additionally sequenced one sample from three *T. angustula* workers collected from a nest at the Instituto de Biociências, Universidade de São Paulo. These workers were collected while returning to their colony from foraging trips. Total

RNA was extracted from each individual separately using the RNeasy® Mini Kit (Qiagen). RNA extractions from individuals of the same nest were then pooled (3 individuals/pool) to reduce individual variation expression within the colonies. These pools are hereafter referred to as samples. In summary, three samples (one per colony) of *L. limao*, *N. testaceicornis* and *S. depilis*; and one sample of *T. angustula* were sequenced. Library construction and sequencing were performed by Macrogen (South Korea) using the Illumina® NovaSeq 6000 platform and Illumina® suggested protocols to obtain 100 bp paired-end reads.

Transcriptome assembly

The reads quality analysis was performed using the FastQC 0.11.5 (Andrews 2018). Raw reads were then quality-trimmed using Trimmomatic 0.38 (Bolger et al. 2014) (options: SLIDINGWINDOW:4:30 LEADING:3 TRAILING:3 MINLEN:80 AVGQUAL:30 HEADCROP:14). *De novo* transcriptome assembly was performed independently for each species using Trinity 2.10.0 (Grabherr et al. 2013; Haas et al. 2013), following default parameters, except for the *in silico* read normalization option (set up to 20x maximum coverage). The transcripts resulting from the assemblies were then clustered into SuperTranscripts (Davidson et al. 2017) using the Trinity gene splice modeler script implemented in the trinityrnaseq toolbox (Grabherr et al. 2013; Haas et al. 2013). Downstream analyses were performed using the SuperTranscripts datasets. Quality parameters from SuperTranscripts were analyzed with Transrate 1.0.3 (Smith-Unna et al. 2016) and BUSCO 5.2.2 (Simão et al. 2015).

Annotation and orthologs identification

SuperTranscripts were annotated with Annocript 2.0.1 (Musacchia et al. 2015) using the UniProt Reference Clusters (UniRef90) (Suzek et al. 2015) and

UniProtKB/Swiss-Prot (Bairoch 1996) databases from February 2021. SuperTranscripts identified as possible contaminants (*i.e.*, those annotated to Acari, Alveolata, Archaea, Bacteria, Fungi, Rhizaria, Rhodophyta, Viridiplantae and Viruses) were removed from the final data set using custom scripts (https://github.com/PauloCseri/Annotation.git). Amino acid sequences based on the SuperTranscripts open reading frames (ORFs) generated by Annocript were used to identify orthologs among all species using Orthofinder 2.1.1 (Emms and Kelly 2015, 2019). For each orthogroup inferred by Orthofinder, only a one-to-one ortholog sequence per species was selected based on the best Blastp alignment (BLAST 2.9.0+) (Altschul 1997; Camacho et al. 2009) with default parameters.

Differential expression analyses

Differential expression (DE) analyses were performed using the data set of one-to-one orthologs of each species. To estimate the expression levels, the paired reads were mapped to the species correspondent set of ortholog sequences using Bowtie 2.3.5.1 (Langmead and Salzberg 2012) and with RSEM 1.3.3 (Li and Dewey 2011). The identification of differentially expressed orthologs between *L. limao* and all other stingless bee species was performed using two methods: (1) using the TMM normalized counts (Robinson and Oshlack 2010) analyzed by edgeR 3.34.0 (Robinson et al. 2010); and (2) using the scale based normalized (SCBN) counts (Zhou et al. 2019) analyzed by NOISeq 2.36.0 (Tarazona et al. 2015). The SCBN method is a count normalization strategy optimized for cross-species DE analyses (Zhou et al. 2019) implemented in the Bioconductor package SCBN 1.10.0. Only significant differentially expressed transcripts (at a cut-off of $|Log2FC| \geq 2$, adjusted p-value < 0.05) identified by using both methods were considered DE orthologs. Lastly, we compared all pairwise differential expression results to identify the set of orthologs consistently differently expressed between *L. limao* and all pollen-collecting species.

GO functional analysis

Among the DE orthologs identified between *L. limao* and all the other species, we identified GO-enriched terms using the Bioconductor package TopGO 2.20.0 (Alexa and Rahnenfuhrer 2020). Redundant enriched terms for Biological Process (BP) were summarized in a two-level hierarchical GO set using REVIGO web server (Supek et al. 2011) and these hierarchical sets were represented in a chart generated using the CirGO 2.0 software (Kuznetsova et al. 2019).

Results

Transcriptome assembly and annotation

The RNA sequencing of *L. limao* resulted in 254,713,432 raw reads, that of *N. testaceicornis* in 261,049,812, and for *S. aff. depilis* 259,553,460 reads were generated. The number of reads decreased to 182,096,230, 187,198,886, and 185,326.996, respectively, after cleaning, For *T. angustula*, 89,081,896 new raw reads were sequenced, in addition to 118,318,606 cleaned reads from a previous study (Araujo and Arias 2021), resulting in a final set of 182,707,804 cleaned reads. The main quality parameters associated with each transcriptome assembly are summarized in Table 1.

Table 1. Main quality parameters for the transcriptome assemblies of *Lestrimelitta limao*, *Nannotrigona testaceicornis*, *Scaptotrigona aff. depilis*, and *Tetragonisca angustula*.

Metric	***L. limao***	***N. testaceicornis***	***S. aff. depilis***	***T. angustula***
Total transcripts assembled	97,316	127,496	137,488	152,927
Number of clustered SuperTranscripts	57,125	82,622	91,872	85,001
SuperTranscripts ≥ 1,000 nt	11,023	13,839	16,366	20,346
p fragments mapped[a]	0.92	0.91	0.92	0.91
p bases uncovered[b]	0.02	0.01	0.01	0.02
Transrate optimal score[c]	0.48	0.48	0.50	0.52
Complete BUSCOs (%)	88.6	89.2	90.6	92.6
Missing BUSCOs (%)	7.6	6.6	6.2	4.6

[a] proportion of read pairs that mapped successfully.
[b] proportion of bases that were not covered by any read.
[c] this discrete score represents the accuracy and completeness of the assembly based on the assessment of alignments between contigs and reads. The minimum score is 0, while the maximum is 1.0. For a description of the components used in the calculation of this metric, see Smith-Unna et al. (2016).

The number of transcripts assembled for *L. limao* was lower in comparison to the other species. It is worth mentioning that the amount of RNA-seq reads was similar for all of them. Accordingly, after the annotation step and excluding potential contaminants (Supplementary Material 1), *L. limao* was the species with the lowest number of Super-Transcripts returning significant blast hits with protein-coding gene sequences from the UniRef90 database, 24,859 (43,5% of total SuperTranscripts). For *N. testaceicornis*, *S. aff. depilis*, and *T. angustula* this number was, respectively, 32,452 (39,2%), 38,273 (41,65%), and 33,596 (39,5%). Moreover, *L. limao* also presented a considerably lower number of potentially contaminating sequences (379) against *N. testaceicornis* (13,802), *S. aff. depilis* (14,414), and *T. angustula* (9,096). Most of the potential contaminants obtained for *N. testaceicornis*, *S. aff. depilis*, and *T. angustula* had significant blast hits with plant sequences (13,588, 14,033, and 8,467, respectively), particularly with plants of Fabaceae and Myrtaceae families, which is likely due to their pollen-collecting behavior. The cleptobiotic species L. limao, on the other hand, presented only 379 SuperTranscripts identified as potential contaminant sequences, of which 87 were from plants, and the remainder attributed mainly to fungi and bacteria.

Differential orthologs expression

Orthofinder identified 44,968 orthogroups, of which 8,313 were common to all four species (Figure 1A). *L. limao* showed the smallest amount of species-specific orthogroups (140) and only 49 of those included at least one transcript annotated. We retrieved 7,482 one-to-one orthologs occurring in all species. This set of orthologs was used for differential expression analyses. Finally, we identified a total of 175 orthologs commonly differentially expressed between *L. limao* and all the other three species studied (Figure 1B), most of which, were downregulated in *L. limao*.

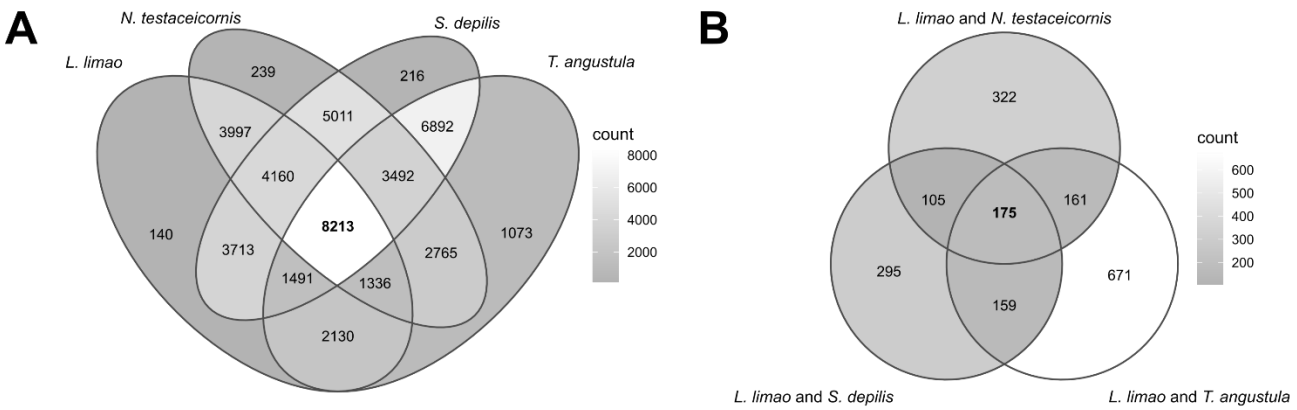

Figure 1. Analysis of the orthologs identified for the species *Lestrimelitta limao*, *Nannotrigona testaceicornis*, *Scaptotrigona aff. depilis* and *Tetragonisca angustula*. (A) Venn diagram representing the sets of orthogroups shared between/among species. (B) Venn diagram representing the sets of orthologs differentially expressed (DE) between/among species. Color scales represent count numbers ranging from lowest (dark gray) to highest (white).

We conducted an enrichment analysis of Gene Ontology (GO) terms from the Biological Processes (BP) category identifying 26 enriched BP terms among the differentially expressed orthologs. The redundant BP terms were summarized in a two-level hierarchical list for visualization (Figure 2). The orthologs annotated to these terms are shown in Table 2. Top enriched terms were related to the *transmission of nerve impulse*, *maintenance of presynaptic active zone structure*, and *positive regulation of calcium ion transport*.

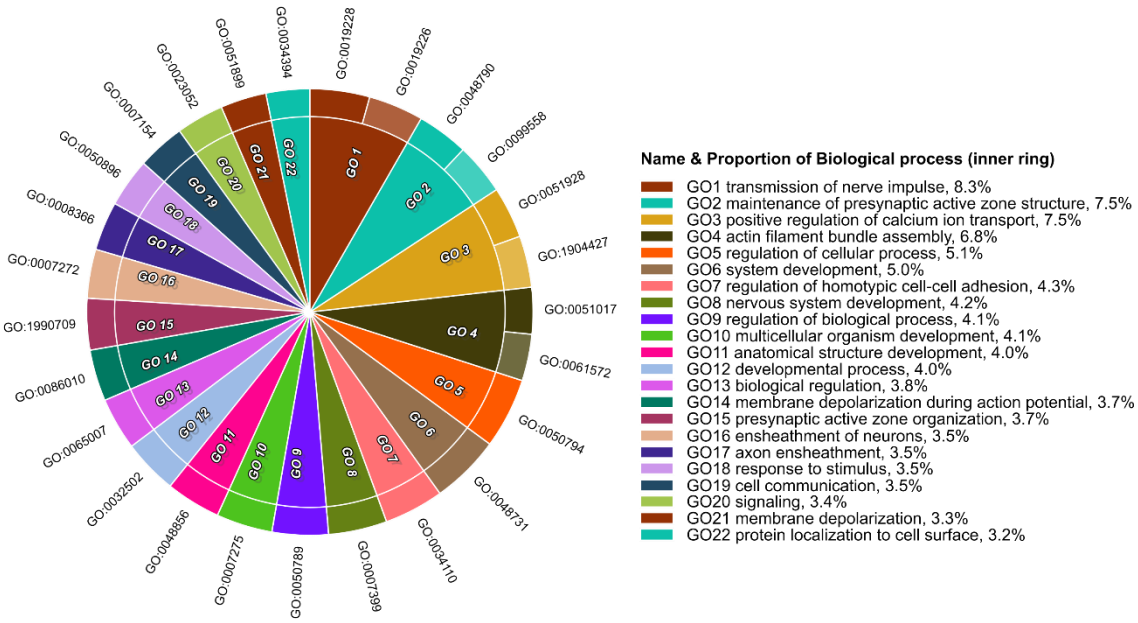

Figure 2. Summarized GO Biological Process terms enriched in the differentially expressed orthologs between the cleptobiotic *L. limao* and the pollen-collecting Meliponini species *Nannotrigona testaceicornis*, *Scaptotrigona depilis* and *Tetragonisca angustula*. The legend next to the chart represents the color, name, and proportion of the GO parents for each two-level hierarchical set of child terms that were enriched.

Table 2. Differentially expressed orthologs between the cleptobiotic *L. limao* and the pollen-collecting Meliponini species *Nannotrigona testaceicornis*, *Scaptotrigona depilis* and *Tetragonisca angustula* annotated to the top enriched Biological Process GO term. The orthologs are represented by the ID of their respective orthogroups. Expression patterns of orthologs are indicated by arrows: ↑ (upregulated) and ↓ (downregulated). The genes and respective origin species from whose genes returned a significant hit (based on the Swiss-Prot database annotation) to each orthogroup are also described.

Orthogroup	Swiss-Prot homologs (organism)	Expression	Putative function
	Peroxidase		
OG0001295	*Duox* (*Drosophila melanogaster*)	*L. limao* ↑ Pollen-collecting ↓	Production of reactive oxygen species (ROS) and potential role in defense against pathogens (Kawahara et al. 2007; Azzouz-Olden et al. 2018).
	Peptidase		
OG0003146	*PAN2* (*D. melanogaster*)	*L. limao* ↑ Pollen-collecting ↓	Involved in mRNA degradation, and in hencing the regulation of transcript abundance (Parker and Song 2004; Wahle and Winkler 2013).
	Uracil-DNA glicosilase		
OG0010914	*TDG* (*Homo sapiens*)	*L. limao* ↑ Pollen-collecting ↓	Repair of damaged DNA by removing thymines and uracils that have been mispaired with guanines (Hardeland et al. 2001).
	DNA helicase		
OG0009618	*MCM3* (*H. sapiens*, *Pongo abelli*)	*L. limao* ↑ Pollen-collecting ↓	Essential in the initiation and elongation phases of chromosome replication (helicase function) (Li et al. 2011).
	Sodium channel		
OG0001240	*para* (*D. melanogaster*)	*L. limao* ↓ Pollen-collecting ↑	Postsynaptic excitatory potential regulation; Potential role in mechanosensory behavior (Engel and Wu 1994; Xiao et al. 2017).
	Distrofin		
OG0003765	*Dys* (*D. melanogaster*)	*L. limao* ↓ Pollen-collecting ↑	Control of neuromuscular synaptic homeostasis (van der Plas et al. 2006); modulation of the microRNA profile (Marrone et al. 2012).
	Glucose-6-phosphate dehydrogenase		
OG0000574	*G6PD*/*Zw* (*H. sapiens*, *D. melanogaster*)	*L. limao* ↓ Pollen-collecting ↑	Role in pentose phosphate pathway and redox balance (Legan et al. 2008; Stincone et al. 2015).
	Serine-threonine phosphatase		
OG0000759	*Ppp3ca*/*Pp2B-14D* (*Rattus norvegicus*, *H.* sapiens, *Bos taurus*, *D. melanogaster*)	*L. limao* ↓ Pollen-collecting ↑	Regulates diverse processes like immunity, sleep, circadian rhythms, and female meiosis (Dijkers and O'Farrell 2007; Nakai et al. 2011; Takeo et al. 2012; Kweon et al. 2018).

	ATPase		
OG0000773	*ATAD3A*/*bor* (*H. sapiens*, *Xenopus tropicalis*, *D. melanogaster*)	*L. limao* ↓ Pollen-collecting ↑	Regulation of the activity and morphology of mitochondria (Gilquin et al. 2010; Harel et al. 2016).
	RING-box protein		
OG0000879	*rbx1*/*RNF7*/Roc1a (*Salmo salar*, *Mus musculus*, *H. sapiens*, *D. melanogaster*,)	*L. limao* ↓ Pollen-collecting ↑	Involved in protein degradation via ubiquitination. Roles in *Drosophila* development and neural remodeling (Noureddine et al. 2002; Arama et al. 2007; Wong et al. 2013).
	Transcriptional elongation factor		
OG0000899	*ELP4* (*H. sapiens*, *Xenopus laevis*, *Danio rerio*)	*L. limao* ↓ Pollen-collecting ↑	Implicated in several processes including transcriptional control, α-tubulin acetylation, and tRNA modifications; related to translational efficiency and fidelity (Dalwadi and Yip 2018; Johansson et al. 2018).
OG0002245	*Iws1* (*M. musculus*, *R. norvegicus*)	*L. limao* ↓ Pollen-collecting ↑	Involved in the elongation, processing, and nuclear exportation of mRNA; important for ensuring the efficient translation of mRNA into protein (Yoh et al. 2007; Brès et al. 2008; Pujari et al. 2010).
	Type IV collagen protein		
OG0001022	*COL4A1*/*COL4A2*/*emb-9*/*Col4a1* (*H. sapiens*, *B. taurus*, *Caenorhabditis elegans*, *D. melanogaster*)	*L. limao* ↓ Pollen-collecting ↑	Encodes collagen IV chains, crucial components of the basement membrane (Morrissey and Sherwood 2015). Role in the adequate development of the structure and function of cardiac, skeletal, and visceral muscle cells during morphogenesis (Kelemen-Valkony et al. 2012; Kiss et al. 2016).
	DNA-binding protein		
OG0001379	*SMARCD1*/*Bap60* (*H. sapiens*, *D. melanogaster*)	*L. limao* ↓ Pollen-collecting ↑	*Bap60*/*SMARCD1* homologs mediate interactions between the Brahma complex and species-specific transcription factors, implicating them in a variety of regulatory processes, e.g., immune response to microbial infections, and neurodevelopmental genes regulation (Liu et al. 2016; Nixon et al. 2019).
	Carboxylase		
OG0002309	*PCK2*/*Pepck1* (*H. sapiens*, *D. melanogaster*)	*L. limao* ↓ Pollen-collecting ↑	Encodes an enzyme crucial for gluconeogenesis (Hanson and Patel 2006).

Discussion

Our results revealed that the cleptobiotic species *L. limao* exhibits lower expression levels for the majority of the differentially expressed orthologs, including genes potentially involved in the regulation of neuronal and synaptic activities, as well as other critical cellular functions. While the specific roles of these genes in the context of cleptobiosis remain unclear, certain ortholog groups provided intriguing insights. For example, among the downregulated orthologs in *L. limao*, we identified homologous sequences to members of the cytochrome P450 monooxygenase superfamily. This superfamily, known for its diverse enzymatic functions, includes gene sequences (CYP genes) present in the genomes of almost all living organisms (Werck-Reichhart and Feyereisen 2000). P450 enzymes catalyze a wide range of oxidative transformations, with several of them specialized in the metabolism of endogenous substrates, whereas many others are related to metabolism or detoxification of xenobiotics (Feyereisen 2012). Within arthropods, there are currently six identified CYP clans, the major ones are CYP2, CYP3, CYP4, and mitochondrial, with 99, 156, 101, and 44 subfamilies, respectively (Dermauw et al. 2020). We identified downregulated orthologs homologous to sequences of the CYP2 (*Cyp304a1*) and CYP3 (*Cyp6a14* and *Cyp6a2*) clans. The CYP2 clan, relatively conserved across insects, is associated with key functions (Feyereisen 2012), such as juvenile hormone and ecdysteroids biosynthesis pathways (Bassett et al. 1997; Namiki et al. 2005; Rewitz et al. 2006; Li et al. 2014), and development of sensory structures (Willingham and Keil 2004). The CYP3 clan is the most diverse among arthropods, however, due to gene expansions in particular insect lineages, the distribution of its subfamilies can vary greatly (Dermauw et al. 2020). The CYP6 family, to which *Cyp6a14* and *Cyp6a2* belong, comprises several genes related to the metabolism of xenobiotics and phytochemicals (Feyereisen 2012). In *Tribolium castaneum* (Coleoptera: Tenebrionidae) variants of *Cyp6a14* were observed to increase the detoxification capacity of phytochemicals with

toxic effects isolated from *Bolanthus turcicus* (Caryophyllaceae) (Ercan et al. 2020). In *Aphis glycines* (Hemiptera: Aphididae) it was observed that the transcriptional level of a *CYP6A14*-like increases resistance to a lambda–cyhalothrin pesticide belonging to the group of pyrethroids (Xi et al. 2015). In turn, the heterologous expression of *Drosophila Cyp6a2* is related to resistance to organophosphorus pesticides (e.g., diazinon) (Dunkov et al. 1997). Also, *Cyp6a2* is differentially expressed in *Drosophila* genotypes resistant to dichlorodiphenyltrichloroethane (DDT) (Pedra et al. 2004). Moreover, Johnson et al. (2018) found positive selection signals in bee genes of the CYP6AS subfamily, belonging to the CYP6A family of the CYP3 clan. These genes are related to the metabolism of some flavonoids, phytochemicals characteristic of plant nectar, pollen, and resins, which can be toxic if not metabolized (Liu et al. 2005; Mao et al. 2017). Thus, CYP-mediated detoxification appears to have allowed bees to consume this flavonoid-rich diet (Johnson et al. 2018). It is described that *Lestrimelitta* species does not visit flowers for resource collection, obtaining its food exclusively through the pillaging of nests of other bees (Michener 1946; Sakagami and Laroca 1963a). In fact, our analysis of potential contaminants found a much lower number of sequences associated with plants than in pollen-collecting species. Considering this behavior and our findings, the lower expression of these CYP genes in *L. limao* may be due to the robber workers obtaining food and other nest resources that have already been collected and processed from the other bee species, resulting in less exposure to phytochemicals and other compounds potentially toxic and hence a reduced need for detoxification mechanisms.

Furthermore, a downregulated ortholog related to a putative odorant receptor (significant blast hits with *Or1* from *Apis cerana* and LOC100741725 from *Bombus impatiens*) was also downregulated in *L. limao*. Based on expression data, it is suggested that *AcerOr1* is related to the perception of volatile floral scents (Zhao et al. 2014). Thus, at

first, this result is consistent with the robbing behavior performed by *L. limao* workers as for these bees it is unnecessary to follow chemical cues to obtain food directly from flowers. However, the OR gene family is large and extremely divergent, especially in Hymenoptera (reviewed in Zhao et al. 2014), therefore, it is likely that the identified orthologs have a conserved function in *A. cerana* and *L. limao*. Furthermore, our approach based on the analysis of orthologs is expected to restrict the number of ORs recovered to only conserved proteins, not allowing for the comparison of species-specific ORs expressed in each of the investigated species. Indeed, some ORs, as well as other chemosensory-related genes (CRGs), must play a role in the cleptobiotic behavior of *L. limao*, e.g., intraspecific communication in the organization of attacks (Sakagami et al. 1993; von Zuben et al. 2016). Additionally, it is possible that the cleptobiotic behavior influenced the evolutionary dynamics of these gene families, as it has been proposed for the socially parasitic bumblebees of the subgenus *Bombus* (*Psythrus*), which lost ORs genes compared to the non-parasitic bumblebees (Sun et al. 2021). Still, we were unable – due to the discussed reasons – to properly investigate the involvement of ORs in the behavior of *L. limao*.

L. limao also exhibited downregulated orthologs associated with mitochondrial activity and carbohydrate metabolism, with enriched GO term annotations. One of these was a homolog to the vertebrate *ATAD3A* and the *Drosophila bor* that encodes an ATPase family AAA^+ domain-containing protein. The ATAD3A protein is present exclusively in multicellular eukaryotes and participates in the control of mitochondrial dynamics at the interface between the inner (IM) and outer (OM) membranes (Gilquin et al. 2010). Whereas interactions between IM and OM control several essential mitochondrial functions, the ATAD3 protein is thought to play a role in cell proliferation, metabolic activity, mitochondrial fusion, and fission (Hoffmann et al. 2009; Gilquin et al. 2010; Harel et al.

2016), and in mitochondrial DNA nucleoid organization (Wang and Bogenhagen 2006; He et al. 2007). Furthermore, it was observed that the overexpression of ATAD3A is positively related to the production of elongated mitochondria (Harel et al. 2016), a phenotype that increases the efficiency of mitochondrial ATP production (Gomes et al. 2011). The second ortholog was identified as homologous to human *PCK2* and *Drosophila Pepck1*, which encode phosphoenolpyruvate carboxykinase, an enzyme essential in gluconeogenesis (Hanson and Patel 2006). Gluconeogenesis is an essential process for maintaining sugar homeostasis in insects, producing glucose from stored glycogen, in addition to being the initial step in the generation of trehalose (Miyamoto and Amrein 2017), a disaccharide presents in various organisms, including bacteria, fungi, plants and invertebrates (Elbein 2003). Trehalose is the major sugar in the hemolymph of many insects (Matsuda et al. 2015) and is considered an important source to produce ATP energy during flight (Becker et al. 1996) since insect flight is a metabolic process that most demands energy in the animal kingdom (Bretscher and O'Connor 2020). In addition, we also found a homolog to the human *G6PD* and the *Drosophila Zw* downregulated in *L. limao*. *G6PD*/*Zw* encode the Glucose-6-phosphate 1-dehydrogenase, which catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconate coupled to the reduction of NADP+ to NADPH, which is the first and often quoted rate-limiting step in the pentose phosphate pathway (PPP) (reviewed in Stincone et al. 2015). This enzyme also plays an important role in the maintenance of the cellular redox state. (Kletzien et al. 1994; Legan et al. 2008). In this regard, it has been proposed that PPP may operate to reduce the oxidative stress brought on by flying activity, particularly in species whose diets are rich in sugars, as is the case of insects that feed on nectar (Levin et al. 2017). Considering that the expression of these orthologs may be involved with energy demand, and with protection against oxidative muscular damage, especially from flight activity, and that the

overall expression levels of *L. limao* workers were reduced in comparison to the other bees, we hypothesize that the *L. limao* workers sampled in this study were possibly not engaged in high-energy demanding activities. (Hanauer-Thieser and Nachtigall, 1995). We took special care to sample *L. limao* workers when they were returning to their nests based on the behavior of foragers from pollen-collecting species, but we did not sample individuals during raids. The observed expression pattern may undergo a significant change during a raid episode as worker activity is increased, and this could lead to important metabolic changes. Our findings thus imply that the robber bee forage workers have either evolved an overall reduced energetic demand or an additional foraging state in which workers perform only low-demanding energy activities, such as short-distance recognition flights.

In summary, the expression pattern of genes in *L. limao* in comparison to other eusocial bee workers provides valuable insights into the molecular mechanisms associated with its cleptobiotic behavior during foraging, offering avenues for further investigation into the adaptation and ecological dynamics of this unique species.

Conclusions

Lestrimelitta species constitute one of the most notable and well-known examples of obligate cleptobiosis. Despite this, there is still a large gap in knowledge about the genetic factors underlying this behavior. Herein, we provide a starting point for these investigations. Our results demonstrated that most DE orthologs are downregulated in *L. limao* workers, and some of them may reflect specific aspects of obligate cleptobiosis in bees, such as the downregulation of *CYP* genes, which seem to be related to florivory. We also found several DE orthologs that potentially regulate neuronal and synaptic activities in addition to other important cellular processes. Although in this work it was not possible to carry out the functional characterization of most of these orthologs, we

advocate that these genes are potential candidates for future studies involving cleptobiotic bee species. Also, we found evidence that the robber foragers in this study have reduced energetic demands when compared to other eusocial bee workers, suggesting the existence of an alternative foraging state in *L. limao*. The expression profiles of robber bees during raids with tissue-specific resolution may provide additional evidence to delve further into the molecular mechanisms of this behavior.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Author contributions

PCR: Conceptualization, methodology, sampling, investigation, data analyses and interpretation. NSA: Conceptualization, methodology, assistance in data analyses and interpretation, and fund acquisition. MCA: Conceptualization, supervision, fund acquisition. All authors contributed to the preparation of the manuscript.

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