Vitellogenin of the solitary bees *Centris tarsata* and *Centris analis* (Hymenoptera: Apidae): cDNA structural analysis and gene expression

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Abstract – Vitellogenin (Vg) plays a vital role in the reproduction of oviparous species, as the precursor of the major egg storage protein. In bees, most of the studies of this protein rely on honey bees (*Apis mellifera*), and very little is known in solitary species. The solitary bees *Centris tarsata* and *C. analis* were chosen to characterize vg cDNA and better understand its expression. The Vg-deduced amino acid sequences from these bees have typical Vg features found in other hymenopterans. Besides its main site of synthesis, the fat body, transcripts of vg were also observed in ovaries. Males also showed expression of vg, although in lower levels compared with females. During development, vg expression was observed in late larvae, pupae, and female adults. The highest amounts of vg transcripts were observed in egg-laying females. Expression of vg increased rapidly in a few hours after female adult emergence, which indicates that its gene expression does not depend on mating to be triggered.

vitellogenin / gene expression / males / ovaries

1. INTRODUCTION

Vitellogenin (Vg) is one of the most important reproduction-related proteins in egg-laying animals, as it is the precursor of the yolk protein vitellin (Vn), the main energy source for develop-

ing embryos (Raikhel and Dhadialla 1992; Sappington and Raikhel 1998). Thus, vitellogenesis is crucial for the reproduction of oviparous animals (Wahli 1988; Carducci et al. 2019). In several species of insects, including social and solitary bees, the ability to initiate vitellogenesis and subsequently promote the growth of the ovaries depends on rates of synthesis of Vg (Hartfelder and Engels 1998; Barchuk et al. 2002; Morandin et al. 2019). Vg is synthesized primarily by the fat body and after synthesis undergoes extensive structural alterations such as phosphorylation, lipidation, glycosylation, among other processes (Raikhel and Dhadialla 1992). It

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may undergo proteolytic processing, and is then transported by hemolymph, and finally endocytosed by specific receptors located on the surface of oocytes, where it is stored as Vn (Raikhel and Dhadialla 1992; Guidugli-Lazzarini et al. 2008; Tufail and Takeda 2008).

Vg is a high-density protein consisting of carbohydrates, a phosphate group, and residues of amino acids covalently bound to phospholipids and diacylglycerol, giving the structure of Vg the denomination of lipoprotein (Engels et al. 1990; Wheeler and Kawooya 1990). Studies on social insects have stated that this protein is multifunctional (Richards 2019). In addition to its role in reproduction, it participates in other functions, such as regulating longevity through its anti-oxidative properties (Seehuus et al. 2006), participating in the immune response by passing information from the queen to its offspring (Salmela et al. 2015; Harwood et al. 2019), and is involved in the energy metabolism of glial cells in the brain (Münch et al. 2015). Additionally, it assists in the transport of molecules, such as sugar, lipids, phosphates, vitamins, zinc, antioxidants, and hormones, which explains its appearance in males (Harnish and White 1982; Sappington and Raikhel 1998).

The complete sequencing of the cDNA of *vg* and expression analyses have been carried out in few Hymenoptera species, and it is the most well-studied in the social bee *Apis mellifera* (Piulachs et al. 2003) from the Apidae family. *vg* expression in *A. mellifera* is dynamic and varies: both during and across developmental stages, and according to sex, hormones, and castes (Barchuk et al. 2002; Piulachs et al. 2003; Guidugli et al. 2005). Vg titers in the hemolymph and its transcripts are much higher in queens than in males and workers (Engels et al. 1990; Barchuk et al. 2002; Piulachs et al. 2003; Guidugli et al. 2005). Gene expression in queens begins when individuals are still in the larval stage, then disappears in the early stages of the pupae, and then reappears in the middle of the pupal stage (Piulachs et al. 2003; Guidugli et al. 2005). *vg* expression in workers and males similarly initiates during the larval stages (with strong expression during the fifth instar), which decreases gradually until it disappears during pupal development, to then reappear during the last

pupal stage in workers and newly emerged males (Barchuk et al. 2002; Piulachs et al. 2003; Guidugli et al. 2005). Within insects, the fact that Vg has been implicated in many functions besides reproduction suggests that male and workers expression of *vg* is related in some way to these other functions (Piulachs et al. 2003). However, the costly production of Vg in workers is also correlated to social behavior (Amdam et al. 2004a; Amdam and Page 2010).

The reproductive division of labor is a hallmark of eusociality, where individuals with differences in physiology, morphology, and behavior are separated into reproductive castes: queens, the reproductive females, and workers, the non-reproductive females (Sumner et al. 2018). It has been suggested that bee eusociality evolved through the developmental decoupling of reproductive and maternal behavior from the ancestral solitary bee reproductive “ground plan” (Amdam et al. 2004a). Vg is a key protein in this process because, in addition to reproduction, it is essential to the division of labor playing an important role in regulating the nurse-to-forager transition (Amdam and Omholt 2003). Therefore, studying solitary species may provide a basis for genetic, physiological, and behavioral underpinnings for evolutionary transitions to sociality. Despite the great knowledge of this protein in other species of insects, very little is known in solitary bees. There is only one study that characterizes *vg* in a solitary bee: *Osmia cornifrons*, belonging to the Megachilidae family (Lee et al. 2015).

Centris (Hemisiella) tarsata Smith 1874 and *Centris (Heterocentris) analis* (Fabricius 1804) are solitary, oil-collecting bees with a broad geographic range that extends from central Mexico to southern Brazil (Moure et al. 2012). They belong to the Apinae subfamily (Apidae family), which comprises solitary (most of the species), primitively social (Bombini), and highly eusocial bees (Meliponini and Apini) (Bossert et al. 2019). Nonetheless, studies have mainly focused on social species, and only one solitary Apidae has had its genome sequenced, annotated, and published (*Habropoda laboriosa*; Kapheim et al. 2015).

Females of *C. tarsata* and *C. analis* build their nests in pre-existing cavities in deadwood, hollow plant stems, or abandoned nests (Michener 2007;

Garófalo et al. 2012), allowing for trap-nests studies (Krombein 1967; Macivor 2017). They are economically significant in pollinator-dependent crops, and the best solitary species candidates for pollination programs in Brazil since they are widely distributed, relatively constant, and abundant in most cavity-nesting bee studies (Garófalo et al. 2004, 2012). In fact, trap-nests occupied by these species in the Neotropics are very common, and after the eggs are laid, immatures can be developed in the laboratory (Garófalo et al. 2004, 2012; Araújo et al. 2016; Lourenço et al. 2020). Molecular studies have indicated that *Centris* is the closest genus to the corbiculate bees (Euglossini, Meliponini, Bombini, and Apini), where eusociality evolved (Martins et al. 2014). As the sister clade of the corbiculate bees, *Centris* is one the best group for interpreting physiological and genomic evolution of Apinae (Martins et al. 2014). These features make these species potential model organisms for solitary bees from the Apidae family for studies on evolution, ecology, behavior, genetics, and physiology.

Here, we report the first molecular information on Vg from *C. tarsata* and *C. analis*. Expression studies were carried out throughout development, across tissues and sexes. The Vg encoding genes were sequenced and used for *in silico* and molecular characterization in these two species. These data were used to characterize Vg structure and expression within these two species as well as to perform cross-species comparisons to interpret our results within the social and solitary context. *Centris* provides a phylogenetically comparison to previous studies on Vg of the eusocial bees (e.g., *A. mellifera* and *Bombus*), in addition, to investigate reproductive physiology inherent to solitary bees independently to phylogenetic relatedness. The current work aims to bridge the gap on the lack of information between the reproduction-related proteins and reproduction physiology in solitary Apinae bees, essential to understanding the evolution of sociality within corbiculate bees. The objectives of this work were to (1) characterize Vg sequence and expression in *C. tarsata* and *C. analis*, (2) investigate similarities of Vg structure and expression in solitary and social bees, (3) determine whether males (sexes) and ovaries (tissues) express vg, and (4)

determine whether age and mating influences on vg expression profiles on adult females (in newly emerged bees and foundress).

2. MATERIAL AND METHODS

2.1. Sample collection

Nests of *C. tarsata* were collected in 2015–2017 from a trap-nest station located in the Juscelino Kubitschek (JK) Campus of the Federal University of the Jequitinhonha and Mucuri Valleys, Diamantina, Minas Gerais, Brazil. Nests of *C. analis* were collected in 2019 from trap-nests located in the Experimental Apiary area of the Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, São Paulo, Brazil. The trap-nests were made of cardboard tubes or bamboo canes (description of the cardboard and bamboo canes for *C. tarsata* in Araújo et al. 2016; Lourenço et al. 2020; description of the cardboard tubes of 5.5 cm length for *C. analis* in Alonso et al. 2012). Using this methodology, about 10 nests/year is occupied by *C. tarsata* (Araújo et al. 2016; Lourenço et al. 2020) and 12 nests/year by *C. analis* (Alonso et al. 2012) in the same areas we sampled, and the number can vary depending on the year. The number of individuals per nest ranged from 2 to 5 for *C. tarsata*, and from 2 to 3 for *C. analis* (Figure S1).

For both bee species, the occupied nests were opened and immatures were individually placed in a 48-cell culture plate, then left in an incubator at 28 °C to develop until the required stages: feeding larvae (weighing 0.060 ± 0.026 g), spinning larvae (non-feeding larvae; weighing 0.171 ± 0.033 g), white-eyed pupae (white eye and unpigmented cuticle), brown-eyed pupae (brown eye and unpigmented cuticle), newly emerged female, and newly emerged male (description of the methodology in Figure S1). As in all solitary bee species, the egg is placed on top of a food mass, and then the foundress closes the cell (Batra 1984). Thus, during transference, the feeding larvae were transferred with the food. Bees were considered newly emerged when they were moving, their wings were completely expanded and dark, and the eyes were green. In four nests, we

also compared the gene expression of two newly emerged females of *C. tarsata* from each nest. The bees were collected at the same time, and thus with few hours of difference in development, which we called older (egg-laid earlier; emerge later) and younger bees (Figure S1). Foundress (egg-laying) females of unknown age were also captured when either returning from the field to their nest or vice versa. The ovaries of foundresses were dissected out in cold DEPC-treated saline (0.9% NaCl), alongside the abdominal carcass carrying fat body cells and cuticular epithelial cells. All samples were kept at -80°C until total RNA extractions.

2.2. Identification of the vitellogenin gene in *Centris tarsata*

The vg partial cDNA sequence of *C. analis* was provided by MMG Bitondi and T Falcon from the raw sequences of RNA-Seq libraries (GenBank BioProject ID PRJNA490324; Falcon et al. 2019). This sequence was deposited in GenBank (GenBank Accession no. BK013350), and used to design several primers for sequencing the *C. tarsata* vg cDNA (Table S1). For the amplification, we first isolated the total RNA from abdomens of newly emerged females using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Samples were treated with DNaseI Amplification Grad (Invitrogen) to eliminate contaminant genomic DNA. For the reverse transcription, we used 3 μg of total RNA, Superscript III (Invitrogen), and Oligo (dT)₂₀ (Invitrogen), according to the manufacturer's instructions. PCR amplifications were done using Taq High Fidelity (Platinum™ Taq DNA Polymerase High Fidelity—Invitrogen), and the reaction conditions were according to the primer's pairs (Table S1). For the 3' region sequencing, a 3' RACE was made. To this end, cDNA was synthesized from total RNA of the carcass (abdomen without ovaries and intestine) of foundresses (extracted using Trizol methodology as described above) using the SuperScript II method and replacing Oligo dT (12–18) with an adapter primer: Adapter primer - 5'- GGCCACGCGTCGACTAGTAC

TTTTTTTTTTTTTTTTTTTTTTTTTTT-3', containing the anchor sequence for the 3' RACE PCR primer (Table S1). After PCRs, the amplified fragments were either purified using a PCR purification kit (PureLink™ Quick Gel Extraction and PCR Purification Combo Kit—Invitrogen) or sequenced directly (2 μl PCR product in a final volume of 10 μl sequencing reaction). The sequencing was done at LaCTAD (UNICAMP-Campinas-SP) and at the Nucleus of Services in Biotechnology (NSB) from the Hemocenter Foundation (USP-Ribeirão Preto-SP), both with Sanger methodology, on ABI 3730XL (Applied Biosystems) and ABI 3500XL (Applied Biosystems) equipment, respectively. The *C. tarsata* cDNA vg sequence was deposited to GenBank under the accession number MW001205.

2.3. In silico analysis of bee vitellogenins

The Artemis platform version 16.0.0 (Rutherford et al. 2000) was used to manually annotate the structural organization of ORFs and putative exon/intron splice sites for vitellogenin of the following bee species: *Apis dorsata* (Ador), *Apis florea* (Aflo), *Apis mellifera* (Amel), *Bombus impatiens* (Bimp), *Bombus terrestris* (Bter), *Duforea novaeangliae* (Dnov), *Exaerete mexicana* (Emex), *Euglossa dilemma* (Edil), *Friesiomellita varia* (Fvar), *Habropoda laboriosa* (Hlab), *Lasioglossum albipes* (Lalb), and *Melipona quadrifasciata* (Mqua). Sequences were retrieved from the Hymenoptera Genome Database (<http://hymenopteragenome.org>), except *F. varia* vg (GenBank BioProject ID PRJNA528016, Freitas et al. 2020) and *C. tarsata* vg (Ctar) (this study). We also used a partial vg sequence from *C. analis* (Cana) (GenBank BioProject ID PRJNA490324, Falcon et al. 2019). Other sequences were retrieved from the NCBI: *Apis cerana* (Acer) (GenBank accession number MG775224.1), *Osmia cornifrons* (Ocor) (AIU68826.1), *Megachile rotundata* (Mrot) (XP_003703020.1), *Bombus ignitus* (Bign) (ACM46019.1), and *Bombus hypocrita* (Bhyp) (ACU00433.1). The manual annotations were done because we observed some inconsistencies

in the automatic annotation of the predicted sequences in the Hymenoptera Genome Database (annotated sequences are in Supplementary data 1). Vg amino acid sequences were imported to the MEGA program version X (Kumar et al. 2018) for building multiple sequence alignments (MUSCLE) and calculating and constructing phylogenetic trees. Phylogenetic analysis was performed using the neighbor-joining method under the Jones-Taylor-Thornton (JTT) matrix-based model (Jones et al. 1992). The rates between sites were treated as a gamma distribution. Bootstrap confidence limit probabilities were estimated from 1000 replications. Vitellogenin amino acid sequence of *Nasonia vitripennis* (Nvit) (XP_001607388.1) and *Pimpla nipponica* (Pnip) (AAC32024.1) were used as outgroups. The Vg tree was visualized using FigTree v1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>). The modular domains of the Vgs were analyzed with the SMART program (<http://smart.embl-heidelberg.de/>) (Letunic and Bork 2017), using SMART mode: Normal, not filling the Sequence ID, and by searching for signal peptides.

The signal sequences of the Vgs were predicted using SignalP 5.0 (<http://www.cbs.dtu.dk/services/SignalP/>), the N-glycosylation sites were predicted using the NetGly 1.0 Prediction Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>), and the isoelectric points (pI) and molecular weights (Mw) were computed using pI/Mw at ExPASy (https://web.expasy.org/cgi-bin/compute_pi/pi_tool). The prediction of protein three-dimensional (3-D) structure was performed using the protein homology/analogy recognition engine v 2.0 Phyre2 server (<http://www.sbg.bio.ic.ac.uk/phyre2/>) (Kelley et al. 2015).

2.4. vg expression for *Centris tarsata* and *Centris analis* using RT-qPCR

For real-time quantitative PCR (qPCR), total RNA was isolated from whole larvae and pupae, from abdomens of newly emerged females and males, and from carcasses (abdomen without ovaries and intestine) and ovaries of foundress females using TRIzol protocol. Each sample corresponded to one individual. Samples were treated with DNaseI Amplification Grad

(Invitrogen) to eliminate contaminant genomic DNA. RNA concentration was determined by absorption at 260 nm using UV spectrophotometry. For reverse transcription, we used 1–5 µg of total RNA of *C. tarsata* and 3 µg of total RNA of *C. analis*, Superscript III (Invitrogen) and Oligo (dT)₂₀ (Invitrogen), according to the suppliers' protocol.

qPCR assays for *C. tarsata* were performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems). Amplification was carried out in a 20 µL reaction volume, containing 10 µL of SYBR Green Master Mix 2× (Applied Biosystems), 2 µL of cDNA (diluted 5×), and 4 pmol of each gene-specific primer, according to the manufacturer's instructions. The PCR conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Specific primers used for *C. tarsata* vg amplification in qRT-PCR assays are in Table S2. Expression measurements of vg were normalized relative to *rpL32* designed for *Centris flavifrons* (GenBank accession number JI020981.1) (primer sequences in Table S2). This gene is a suitable reference gene for normalization in gene expression analysis in Apidae bees (Lourenço et al. 2008; Freitas et al. 2019).

For *C. analis* expression analyses, qPCR assays were performed using the StepOne™ Real-Time PCR System (Applied Biosystems). Amplification was carried out in a 15 µL reaction volume, containing 7.5 µL of SYBR Green Master Mix 2× (Applied Biosystems), 2 µL of cDNA (diluted 5×), and 3 pmol of each gene-specific primer, according to the manufacturer's instructions. PCR conditions were the same as described above. Primers used for *C. analis* vg amplification in qPCR assays are in Table S2. Gene expression normalization was relative to *rpL32* designed for *C. analis* (primer sequences in Table S2). *rpL32* partial sequence was annotated using the raw sequences of RNA-Seq libraries (GenBank BioProject ID PRJNA490324) and deposited in GenBank (GenBank Accession no. BK013351). All primers were designed using Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/>).

The specificity of each pair of primers was checked by melting curve analysis for all qPCR assays (95 °C for 15 s, 60 °C for 1 min, and a continuous raise in temperature to 95 °C at 0.3 °C/s ramp rate followed by 95 °C for 15 s). PCR efficiency values (E) were calculated for each pair of primers and bee species from the given slope after running standard curves and following the formula $E = (10(-1/\text{slope}) - 1) \times 100$ (Table S2).

Data were analyzed according to the comparative threshold cycle (Ct) method, where the relative quantification is given by the amount of a target transcript, normalized to an endogenous reference gene, relative to a calibrator, and primer efficiencies (Pfaffl 2001). To check reproducibility, each biological sample was run in technical triplicate, and outliers were removed according to the default analysis settings from the StepOne™ software v 2.3.

2.5. Protein profile by SDS-PAGE electrophoresis

Protein profiles were performed using hemolymph and ovaries of *C. tarsata* foundresses. Hemolymph was collected after immobilization on ice; a small hole was made in the abdomen (between terga III and IV in the right side) and 4 µL of saline 0.9% was introduced with a micropipette (0.5–10 µL tip). Then, ca. 2 µL of the diluted hemolymph was collected in the same hole using a micropipette. The hemolymph was kept at –20 °C until use. Ovaries were macerated in 15 µL of saline 0.9% and then diluted 2×, 5×, and 10× in double distilled water. As a positive control for protein profile, especially the molecular size of Vg (180 kDa; Wheeler and Kawooya 1990), we also used hemolymph from nurses and newly emerged bees (≤ 24 h old) of *A. mellifera* from a queenright colony from the apiary at Ribeirão Preto. Nurse bees were collected from the brood area, feeding the larvae. For this species, hemolymph was extracted directly from a small hole in the abdomen, and then diluted 10× in saline 0.9%. Aliquots of 0.5 µL diluted hemolymph and 1 µL of the ovarian sample (2×, 5×, and 10× dilutions) were analyzed by SDS-PAGE following Laemmli (1970). Electrophoresis was performed at 15 mA and 7–10 °C, using 10%

polyacrylamide gels (100 × 100 × 1 mm). Gels were stained with silver nitrate (Caetano-Anollés and Gresshoff 1994).

2.6. Statistical analysis

The differences in gene expression throughout development were determined by the non-parametric Kruskal-Wallis and Dunn's post hoc test for multiple comparisons. Gene expression differences between tissues, sexes, and older/younger bees were analyzed using the non-parametric Mann-Whitney test. Analyses were performed with PAST 3.26 software (Hammer et al. 2001). The sample size is given in the relevant figures.

3. RESULTS

3.1. cDNA sequence of *C. tarsata* and *C. analis* vitellogenin, and amino acid sequence comparisons

cDNA sequencing of *C. tarsata* vitellogenin recovered a 5391 bp long sequence with an open reading frame of 5331 bp, encoding a 1777 amino acid fragment, with a calculated molecular weight of 199.5 kDa and theoretical isoelectric point (pI) of 6.67. The amino acid sequence contained a predicted signal peptide (MWLPLTLLVLAGTVSA) at the N-terminus, two N-glycosylation sites, five putative cleavage sites showing the RXXR/S consensus, and the conserved motifs DGKR and GLCG at position 1582–1585 and 1600–1603, respectively, followed by nine cysteines near the C-terminus (Figure S2A). These domains were present in all bees analyzed here (Figure S3). The partial cDNA sequence of *C. analis* vg is 5253 bp long, with an open reading frame of 5219 bp. This partial sequence, encoding a 1739 amino acid fragment, contained a predicted signal peptide (MWLPVTLLVLAATVSA) at the N-terminus, two N-glycosylation sites, four putative cleavage sites showing the RXXR/S consensus, the conserved motifs DGKR and GLCG at position 1584–1587 and 1602–1605, and eight cysteines near the C-terminus (Figure S2B).

During manual annotation of *vg* from sequenced bee genomes (Supplementary data 1), no evidence of *Vg* gene duplication was observed, suggesting that all bees have only one gene for this protein.

Protein-protein BLAST to other bee species showed its highest similarities to solitary bee Vgs (57.25% to *Habropoda laboriosa*, 57.09% to *Osmia cornifrons*, 57.38% to *Megachile rotundata*, and 55.54% to *Duforea novaeangliae*) and lowest similarities to *Melipona quadrifasciata* *Vg* (50.54%). A phylogenetic analysis of the amino acid sequences

revealed that the Vgs of Apidae bees form a cluster, separately from Megachilidae and Halictidae bees, and the *Vg* from *Centris* bees forms a sister group with other Apidae solitary bee, *H. laboriosa* (Figure 1A). The amino acid sequence of *C. tarsata* *Vg* was compared with other 18 bee Vgs, and the main conserved motifs found in Vgs were present (Lipoprotein N-terminal-LPD_N, DUF1943 and Von Willebrand factor type D—VWD; Figure 1B).

High similarities were also observed in 3-D structural prediction between *C. tarsata* and *C. analis* Vgs (primary sequences with 77%

Figure 1. (a) Phylogenetic analysis of amino acid sequences of Vitellogenins of Apidae, Megachilidae, and Halictidae bees. Numbers on the branches of the phylogenetic trees represent bootstrap values for the 1000 bootstrap replicates. *Nasonia vitripennis* *Vg* and *Pimpla nipponica* *Vg* were used as outgroups. (b) Common features and typical domains of the primary structures of Vitellogenins from bee species represented in (a). *Acer*, *Apis cerana*; *Ador*, *Apis dorsata*; *Aflo*, *Apis florea*; *Amel*, *Apis mellifera*; *Bhyp*, *Bombus hypocrita*; *Bing*, *Bombus ignitus*; *Bimp*, *Bombus impatiens*; *Bter*, *Bombus terrestris*; *Cana*, *Centris analis*; *Ctar*, *Centris tarsata*; *Dnov*, *Duforea novaeangliae*; *Emex*, *Exaerete mexicana*; *Edil*, *Euglossa dilemma*; *Fvar*, *Friesiomellita varia*; *Hlab*, *Habropoda laboriosa*; *Lalb*, *Lasioglossum albipes*; *Mqua*, *Melipona quadrifasciata*; *Mrot*, *Megachile rotundata*; *Nvit*, *Nasonia vitripennis*; *Ocor*, *Osmia cornifrons*; *Pnip*, *Pimpla nipponica*. Bootstrap values for each node are shown.

similarities), and these to *A. mellifera* Vg (primary sequences with 55% similarities; Figure 2).

Analyzing the protein profile of hemolymph and ovaries of *C. tarsata*, we observed a ~ 180 kDa band corresponding to Vg (Figure 3). As expected, Vg was the most abundant protein in the ovaries of foundress bees (Figure 3).

3.2. Expression of vitellogenin throughout development, in tissues and between sexes

vg transcripts of *C. tarsata* were quantified in two larval and two pupal stages, newly emerged adult females, and two groups of foundress female adults (Figure 4). The results showed that expression can be detected from spinning larvae and then was continuously detected during the pupal stages and in adulthood. Relatively low expression was observed in larval and pupal stages, and transcript amounts increased up to 98-fold at emergence time when compared with the larval and pupal stages (NE versus LS, Pw, and Pb, Kruskal-Wallis, Dunn's post hoc, $p < 0.001$). Foundress females had 180-fold more expression than newly emerged bees (NE versus FF, Kruskal-Wallis, Dunn's post hoc, $p = 0.025$; Figure 4).

Comparing tissues, *vg* expression was also detected in the ovaries of foundress females of *C. tarsata* with expression 9000-fold lower than in the fat body (Mann-Whitney, $p = 0.012$; Figure 5A). Foundress of *C. analis* showed *vg* expression in the fat body, but no expression was

observed in the ovaries (Figure 5B). Newly emerged males from both bee species also exhibited expression of *vg*. Although *vg* expression was 3.5- and 2-fold higher in females than in males of *C. tarsata* and *C. analis*, respectively, no significant differences were detected (Mann-Whitney, $p > 0.05$; Figure 5A, B).

3.3. Levels of vitellogenin in newly emerged bees from the same nest

Here, we analyzed two newly emerged females of *C. tarsata* from the same nest collected at the same time, and thus with few hours of difference in development (Figure S1). In all four nests, the younger bee (closer to the nest entrance) had more *vg* transcript amount than the older (Mann-Whitney, $p = 0.03$; Figure 6). This result indicates that the amount of *vg* is related to bee developmental time, as the younger bees are in advanced development to emerge earlier.

4. DISCUSSION

In this study, we provided a phylogenetic comparison of Vg across several bee species and described the predicted protein structure of Vg in the solitary bees *C. tarsata* and *C. analis*. Moreover, we analyzed the expression profile of *vg* throughout development, in different tissues and sexes, and its activation after female emergence.

The amino acid sequence of *Centris* contains all three domains, LPD_N, VWD, and DUF1943,

Figure 2. Vitellogenin 3-D structural prediction of (a) *Centris tarsata*, (b) *Centris analis*, and (c) *Apis mellifera* using the Phyre2 server. The best fit was lipovitellin (PBD-ID c1lshA). Image colored by rainbow N → C-terminus.

Figure 3. Silver nitrate-stained 10% SDS-PAGE gel from samples of hemolymph of nurse (Nur) and newly emerged (NE) *Apis mellifera* workers, and hemolymph (H) and ovaries (Ov1: diluted 5×, Ov2: diluted 2×, Ov3: diluted 10×) of *Centris tarsata* foundresses. M: protein molecular weight marker (SIGMA–ALDRICH®/SDS6H2) indicated in kDa. Vitellogenin yolk protein (Vg; ~180 kDa) is indicated in the image.

that are highly conserved in insects (Tufail and Takeda 2008; Shen et al. 2019). Additionally, we

identified the motifs GL/ICG and DGXR at the C-terminus, which are also conserved in insects

Figure 4. Expression of vitellogenin by qPCR throughout the development of *Centris tarsata*: feeding larvae (Larvae_F; $n = 3$), spinning larvae (Larvae_S; $n = 3$), white-eyed pupae (Pw; $n = 3$), brown-eyed pupae (Pb; $n = 3$), newly emerged females (NE; $n = 15$), and foundress females (FF; $n = 5$). Box plots show medians (horizontal lines), 25th to 75th percentiles (boxes), and the minimum value and maximum value (whiskers). The circles indicate internal points. Asterisk indicates no expression. Different letters indicate significant differences ($p < 0.05$).

Figure 5. Expression of *vitellogenin* by qPCR in tissues and sexes of (a) *Centris tarsata* and (b) *Centris analis*. Foundress fat body (FF_Fat Body, *C. tarsata*: $n = 5$; *C. analis*: $n = 3$), foundress ovaries (FF_Ovary, *C. tarsata*: $n = 5$, *C. analis*: $n = 3$), newly emerged males (M_NE, *C. tarsata*: $n = 3$, *C. analis*: $n = 5$), and newly emerged females (F_NE, *C. tarsata*: $n = 7$, *C. analis*: $n = 4$). Box plots show medians (horizontal lines), 25th to 75th percentiles (boxes), and the minimum value and maximum value (whiskers). The circles indicate internal points. Asterisk indicates no expression. Different letters indicate significant differences ($p < 0.05$).

(Tufail and Takeda 2008). These conserved domains and motifs were observed in the Vg from all

19 bees analyzed here. Molecular evolution analyses of *A. mellifera* Vg revealed that the N-

Figure 6. *vitellogenin* expression by qPCR of newly emerged female sisters (older and younger) of *Centris tarsata* from four different nests ($n = 4$). Box plots show medians (horizontal lines), 25th to 75th percentiles (boxes), and the minimum value and maximum value (whiskers). The circles indicate internal points. Different letters indicate significant differences ($p < 0.05$).

terminal domain is resistant to change, while nonsynonymous mutations are enriched of the remaining part of the protein, which is associated with lipid binding (Kent et al. 2011). These results suggested that the N-terminal domain is central to conserved function in reproduction, while the remaining protein has been freer to evolve, and that these polymorphisms can alter ligand-binding properties of Vg (Havukainen et al. 2011; Kent et al. 2011). However, selective pressure remains to be investigated on Vg between solitary and social species.

It is interesting to note that the amino acid sequence (BLASTp) of the Vg from *C. tarsata* was more similar to that more distantly related solitary bees than to that of more closely related social Apidae. However, the inferred phylogenetic tree (gene tree) resulted in *Centris* as the sister group of corbiculate bees, and the overall topology was very similar from molecular bee phylogenies (Cardinal and Danforth 2013; Romiguier et al. 2016; Bossert et al. 2019).

We observed that *vg* expression in *C. tarsata* began in spinning larvae and it was maintained until the adult phase, albeit at very low levels. Due to its relevance to the social organization (Amdam et al. 2003; Nelson et al. 2007), *vg* has mainly been investigated in the social bees: *A. mellifera* (Barchuk et al. 2002; Piulachs et al. 2003; Guidugli et al. 2005), *A. cerana* (Zhang et al. 2017), *Frieseomelitta varia*, *Melipona quadrifasciata*, *Scaptotrigona postica* (Dallacqua et al. 2007), *B. ignitus* (Lee et al. 2009), and *B. hypocrita* (Li et al. 2010). However, its temporal expression in related solitary bees has largely neglected (with the exception of: Lee et al. 2015; *O. cornifrons*). Larval *vg* expression is likewise understudied. Larval *vg* transcripts have been previously only been detected in the eusocial *A. mellifera* (Guidugli et al. 2005) and *A. cerana* (Zhang et al. 2017), yet never before in a solitary bee (Lee et al. 2015). Detection of *vg* during the larval stage in *A. mellifera* (Guidugli et al. 2005), *A. ceranae* (Zhang et al. 2017), and *C. tarsata* (this study) may reflect its role as a multifunctional protein during this stage and may suggest a shared feature for the Apidae family. Storage proteins are ecologically

important gene products playing a role in diapause (Hunt et al. 2007). In solitary bees, this arrest of development occurs mainly during the last larval instar (prepupal stage), and this developmental diapause has been lost in social bees (Santos et al. 2019). The shift from developmental diapause in the solitary ancestor to adult, reproductive, or absence of diapause in social lineages suggests a role of diapause in the evolution of sociality in bees (Santos et al. 2019). In Apidae, Centridini bees comprise only solitary bees being a sister group of the corbiculate bees (Euglossini, Bombini, Meliponini, and Apini; Bossert et al. 2019) where sociality evolved as well as different forms of diapause (adult, reproductive, or absence of diapause). Prepupal diapause was observed in *C. tarsata* during the dry season (Aguiar and Garófalo 2004). Although *O. cornifrons* is a solitary bee, diapause occurs in the adult stage, and *vg* was continuously expressed during diapause stage but not in larvae or prepupae (Lee et al. 2015). These results suggest that *vg* expression in larva may be a retained evolutionary trait from the Apidae ancestor as an important storage protein for diapause. Therefore, more studies of the expression of *vg* during larval stages in bees with different social levels of behavior are needed. Pupal expression of *vg* was observed at lower levels in the last and middle pupal stages in workers and queens of *A. mellifera*, respectively (Barchuk et al. 2002; Piulachs et al. 2003; Guidugli et al. 2005). In workers and queens of *B. hypocrita*, *vg* was detected during all pupal stages, with a gradual increase from initial to late pupal stages (Li et al. 2010); however, in the congeneric bee *B. ignitus*, *vg* was first detected only in the late pupal stage (Lee et al. 2009). We did not investigate *vg* expression in the late pupal stage, but the detection in pupae, especially in increased amounts near the time of bee emergence, may be important for preparing females for the onset of reproduction. In fact, *vg* expression in egg-laying social bees (queens and workers; Piulachs et al. 2003; Li et al. 2010) or solitary bees (Lee et al. 2015 and this study) are much higher, consistent with its primary function as an egg yolk protein.

Comparing the expression of *vg* between ovary and carcass (including the fat body), levels were higher in the latter for both *C. tarsata* and *C. analis*. This result was similar to that obtained in *A. mellifera* (Guidugli et al. 2005) and *A. cerana* (Zhang et al. 2017), which also showed that *vg* transcript levels were higher in fat body tissue. In fact, the fat body of insects is the main organ of the production of *Vg* (Bellés 2004), although other tissues may express the gene encoding *Vg*, such as ovaries (Guidugli et al. 2005). Traces of *vg* expression were only observed in the ovaries of *C. tarsata*.

The presence of *vg* expression in *C. tarsata* and *C. analis* males was also observed, although in smaller amounts, as observed in all studied male bees so far (Piulachs et al. 2003; Guidugli et al. 2005; Dallacqua et al. 2007; Li et al. 2010). Expression in males possibly relates to *Vg* having many functions in insects other than reproduction, i.e., it is used to transport molecules such as sugar, lipids, phosphates, vitamins, and hormones, as well as participating in immune functions as a zinc carrier (Sappington and Raikhel 1998; Amdam et al. 2004b).

Mating has a profound effect on social Hymenoptera, where mated queens produce high amounts of *vg* in ants (Tian et al. 2004; Chérasse et al. 2019) and bees (Kocher et al. 2008). Moreover, mating accelerates queen oogenesis in social bees such as in *Melipona quadrifasciata* (Martins and Serrão 2004; de Souza et al. 2007; Tanaka and Hartfelder 2009) and *A. mellifera* (Tanaka and Hartfelder 2004; Tanaka et al. 2006). Alternately, mating in the solitary bee *Osmia bicornis* does not affect ovary activation or the rate of reproduction over time (Van Eeckhoven and Duncan 2020). The authors using morphology (oocyte volume and position) observed that the rate of oogenesis increased over time in both mated and unmated females, suggesting that oogenesis initiates and accelerates regardless of mating status (Van Eeckhoven and Duncan 2020). Supporting this observation, *vg* expression increases in the first hours after emergence, reaching its maximum in 72 h in another solitary bee, *O. cornifrons* (Lee et al. 2015). This increase in *vg* expression may lead to the maturation of the

oocytes even in unmated females. For instance, the first fully mature oocytes were observed 96 h after *O. bicornis* emergence (Van Eeckhoven and Duncan 2020). Here, we studied sister females, i.e., females from the same nest, that were collected at the same time, but with differences in maturation: older females were located towards the back of the nest and were less mature than their sisters (younger females). As the less mature has a difference of a few hours from the more mature ones, the higher *vg* expression observed in more mature females indicates that the increase of *vg* occurs rapidly (on the order of hours), even in unmated females, which reinforces the idea that mating does not influence the onset of oogenesis nor its acceleration in solitary bees as it does in social Hymenoptera (Van Eeckhoven and Duncan 2020).

Within the Apidae, diverse levels of sociality are found, making it a critical group for studying sociality. The *Vgs* of social species are better studied, but this is the first work characterizing the expression profile of *vg* in solitary Apidae. Its characterization is important to establish the relation of *Vgs* of bees, and ultimately to examine their relation to sociality.

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AUTHOR CONTRIBUTIONS

APL designed the study; APL, JCA, CPC, JTF, ACD, and NPG performed experiments and analysis; APL, JCA, ZLP, and CPC interpreted the data, wrote the paper, and participated in the revisions of it. Funding

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COMPLIANCE WITH ETHICAL STANDARDS

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

Vitellogénine des abeilles solitaires *Centris tarsata* et *Centris analis* (Hymenoptera : Apidae) : analyse structurelle de l'ADNc et expression des genes.

Vitellogénine / expression génétique / mâle / ovaires.

Vitellogenin der solitären Bienen *Centris tarsata* und *Centris analis* (Hymenoptera: Apidae): Analysen der cDNA-Struktur und der Genexpression.

Vitellogenin / Genexpression / Männchen / Ovarien.

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