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**Research article**

# Vitellogenin gene expression in stingless bee workers differing in egg-laying behavior

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**Abstract.** In stingless bee species, workers regularly lay eggs even in queenright colonies, but a variety of patterns ranging from a high frequency to a complete absence of laying workers has been recognized. We investigated whether these differences could be linked to alternative expression patterns of vitellogenin (Vg), the major component of egg yolk. In *Frieseomelitta varia* workers, which never lay eggs, and in *Melipona scutellaris* and *Scaptotrigona postica* that frequently participates in egg-laying activity, we found discrepant developmental patterns of Vg transcript and of the corresponding protein. Interestingly, only in the permanently sterile *F. varia* workers, Vg protein was found to be constitutively present throughout pupal and adult stages. In workers as well as in males of this species, the quality of the alimentary diet shaped the levels of Vg transcript. In conjunct, our data suggest differences in the regulatory mechanisms underlying *vg* gene expression in these stingless bee species, and point to alternative roles for Vg, in addition to its essential function in vitellogenesis.

*Keywords:* Vitellogenin, stingless bees, *Frieseomelitta varia*, *Scaptotrigona postica*, *Melipona scutellaris.*

## Introduction

In insects, the major yolk protein, vitellogenin (Vg), is synthesized in large scale in the fat body, released into hemolymph, and taken up by developing oocytes to be consumed throughout embryogenesis (Engelmann, 1979; Raikhel and Dhadialla, 1992). Vg is downstream of an endocrine signaling chain that in general is triggered by specific environmental factors or conditions, frequently related to food intake, which stimulate the brain and endocrine glands for hormone production that ultimately regulates the biosynthesis of this protein (Yin et al., 1990).

Vg has been traditionally used as an adequate parameter for evaluating insect female fertility. In the highly social *A. mellifera* honey bee, the huge reproductive capacity of the queen was linked to extremely high rates of Vg biosynthesis that is used in the production of up to 2,000 eggs per day. Vg first appears in the hemolymph of queens shortly before adult emergence, and is kept at high titers throughout their life. Young workers also accumulate a considerable amount of Vg in hemolymph that obviously represents only a fraction of that found in queens (Engels et al., 1990; Barchuk et al., 2002). Even so, as part of the social regulation of reproduction, the worker caste is maintained in a functionally sterile condition due to the repressive queen dominance. Workers then are concentrated in raising queen-laid brood, in addition to attend other needs of the colony. However, when the queen dies or fails in making use of its prevailing reproductive dominance, the honey bee workers may intensify Vg biosynthesis and lay eggs (Engels, 1974; Fluri et al., 1982). Because they are unable to mate, their eggs will give rise to haploid males.

In contrast to honey bees, stingless bee workers frequently lay their own eggs in addition to assist those laid by the physogastric queen. Although laying workers occur widely among stingless bee species, a high diversity in relation to the frequency of worker participation in egg laying activity has been found (Sakagami et al., 1963; Sakagami and Zucchi, 1963; Engels and Imperatriz-Fonseca, 1990). In *Scaptotrigona postica*, for example, workers actively lay eggs that serve up to feed the queen (trophic eggs), or originate males (reproductive eggs) (Sakagami and Zucchi, 1963; Beig, 1972). *Friesella schrottkyi* workers lay a low proportion of reproductive,

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**Table 1.** External morphological characters used to stage developing pupae of *F. varia*, *M. scutellaris* and *S. postica* stingless bee species.

| Pupal stage | Eye coloration | Body cuticle |
|---|---|---|
| Pw | White | Unpigmented |
| Pp | Pink | Unpigmented |
| Pb | Brown | Unpigmented |
| Pbl | Dark brown | Pigments first appearing in appendages |
| Pbm | Dark brown | Pigmented appendages; Light-pigmented thorax and abdomen |
| Pbd | Dark brown | Dark-pigmented appendages; Light-pigmented thorax and abdomen |

but not trophic eggs (Imperatriz Fonseca and Kleinert, 1998). *Leurotrigona mueleri* workers produce eggs only in the absence of the queen (Sakagami, 1982). An extreme case is exemplified by *Frieseomelitta varia* workers that never lay eggs because their ovaries enter in a degenerative process of programmed cell death yet before adult emergence (Boleli et al., 1999).

We hypothesized that this diversity of egg-laying patterns, which deviate in many aspects from that found in the honey bee, could be linked to differences in *vg* gene expression. We then examined the expression of this gene, and of its corresponding protein, in species of stingless bees differing in relation to egg-laying behavior. The following species were chosen as representative of variant patterns: *S. postica* and *Melipona scutellaris* workers, which participates in egg-laying activity (Beig, 1972; Engels and Engels, 1977; Tóth et al., 2002), and *F. varia* workers that never lay eggs (Boleli et al., 1999). Vg transcript abundance was determined using semiquantitative RT-PCR. Concomitantly, the presence of Vg in hemolymph was checked by SDS-PAGE and Western blot. Yet, we elected *F. varia* to study the nutritional control of *vg* expression. In the honey bee, pollen consumption and the consequent increase in Vg titer (Bitondi and Simões, 1996), and *vg* transcript (Guidugli-Lazzarini, 2006) ensure vitellogenesis and egg laying in the eventuality of queen loss. In stingless bee species where workers participate in egg-laying activity, the same type of nutritional control is expected to occur. We wanted to know whether in *F. varia* workers, which will never lay eggs, such type of nutritional regulation is also operating.

## Material and methods

*Stingless bees*

*S. postica* and *F. varia* workers at the pupal and adult stages were collected from colonies kept in São Paulo University in Ribeirão Preto, SP. *M. scutellaris* workers (pupae and adults) were obtained from colonies maintained in the apiary of the Federal University in Uberlândia, MG. Pupae were staged using eye and exoskeleton pigmentation as criteria (Table 1). Adult workers were classified as newly emerged, or according the task they were performing: nursing brood (nurse) or collecting pollen (forager). Vg mRNA and protein abundances were determined for pupae and adults, using semiquantitative RT-PCR and Western blot, respectively.

To study the influence of dietary proteins on Vg transcript abundance, two groups of fifty newly emerged *F. varia* (workers and males) were separately confined in 8×11×13 cm screened wooden cages where they were maintained from the emergence to the 12$^{th}$ day of adult life. Cages were maintained in an incubator, under conditions of temperature and relative humidity similar to those detected in hives (28 °C, and 80%). Bee groups were fed either on a diet prepared with processed pollen (beebread, collected from honey bee colonies) and powdered sugarcane at the proportion of 60%, or only on 60% sugar in water. Water was given *ad libitum* to both groups. At the 6$^{th}$ and 12$^{th}$ days, total RNA was isolated from the abdomen of workers and males fed on the different diets, and used for semiquantitative RT-PCR.

*RNA extraction*

Stingless bees at the pupal and adult stages were used as RNA source. Gut was discarded, and the abdominal integument (cuticle and subjacent fat body) was used for total RNA extraction in 1mL TRIzol® (Invitrogen), according to manufacturer instructions. RNA was ressuspended in RNAse-free water, and stored at − 80 °C. Each RNA sample was prepared with the abdominal integument from 5–8 pupae or 6 adults in the same developmental stage. At least 2 replicates were made for each stage.

*Isolation and sequencing of partial cDNAs for Vg using semi-quantitative RT-PCR*

RT-PCR was performed using primers (forward: 5′-CGA CTC GAC CAA CGA CTT C-3′, and reverse: 5′- ACG AAA GGA ACG GTC AAT TCC-3′), designed on the basis of *vg* gene sequences from *A. mellifera* (GenBank accession number: AJ517411).

Preceding cDNA synthesis, total RNA samples were treated with DNase (RNAse free) for 50 min at 37 °C to eliminate contaminant DNA. This was followed by incubation at 65 °C for 15 min to inactivate DNase. First-strand cDNA was synthesized using SuperScript II reverse transcriptase, oligo ($dT_{12\text{-}18}$) primer (Invitrogen), and 1μg of total RNA. Aliquots of first-strand cDNAs were employed in PCR reactions using PCR Master Mix (Eppendorf) and *vg* primers. The thermal cycling program consisted of 2 min at 94 °C followed by 36 cycles of 30 s at 94 °C, 1 min at 58 °C, 1 min at 72 °C and a final extension step at 72 °C for 10 min. Number of cycles was carefully tested to avoid saturation.

The PCR products were analyzed by electrophoresis in ethidium bromide stained agarose gels (1%). A fragment of 500 bp corresponding to the Vg mRNA was purified (PerfectPrep Gel CleanUp, Eppendorf), and cloned using TOPO TA Cloning kit (Invitrogen) or pGEM®-T Easy (Promega). Insert-containing plasmids were sequenced using M13-reverse and M13-forward universal primers. DNA sequencing was performed by the dideoxy sequencing method, using a Big-dye terminator v3.0 Cycle Sequencing Ready Reaction for an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

The nucleotide sequences obtained were translated using DNA-Protein translate tool from ExPaSy proteomics server website (http://www.expasy.org). The deduced amino acid sequences of Vg from the stingless bee species were aligned with Vg sequence from *A. mellifera* by means of ClustalW.

### *vg gene expression*

*vg* gene expression was evaluated by semi-quantitative RT-PCR using total RNA from pupal and adult stages. RT-PCR products were run on agarose gels stained with ethidium bromide. An *A. mellifera actin* gene (GenBank accession number AB023025) was used to control cDNA loading and to correct for differences in cDNA amounts. *actin* gene has been proved to be constitutively expressed in the honey bee at all developmental stages (unpublished data), and has been extensively used in our laboratory as a housekeeping gene. The primers used for *actin* gene amplification were: forward (5′-TGC CAA CAC TGT CCT TTC TG-3′) and reverse (5′-AGA ATT GAC CCA CCA ATC CA-3′), and the thermal cycling program was as follows: 2 min at 94 °C, 30 cycles of 30 s at 94 °C, 45 s at 60 °C, 1 min at 72 °C and a final extension step at 72 °C for 7 min. Control reactions without cDNA or superscript in the RT-PCR procedures were also routinely performed in order to test for genomic DNA contamination.

### *Hemolymph collection*

Hemolymph from stingless bee workers (pupae and adults), and from *A. mellifera* (used for comparison) was rapidly collected using glass microcapillary. Because of the low volume obtained from each individual, hemolymph from 5–10 pupae or 15–20 adults at the same stage was pooled. Two replicates were made for each stage. Samples were centrifuged at 2,000*g* for 2 min at 10 °C. To prevent oxidation (melanization), glass microcapillaries were washed in distilled water containing a few crystals of phenylthiourea, immediately before hemolymph collection. To avoid protease action, 2 µl of a cocktail of inhibitors (0.05 mg/ml Soybean Trypsin Inhibitor, Lima bean Trypsin Inhibitor and Leupeptin; 0.1 M Benzamidine) was added to the hemolymph samples that were subsequently stored at −20 °C. After centrifugation, aliquots of the supernatants were separated for protein quantification using bovine serum albumin in standard curves (Bradford, 1976). Samples containing 5 µg proteins were used for SDS-PAGE and Western blot.

### *SDS-PAGE*

Hemolymph proteins were separated by electrophoresis performed according to Laemmli (1970), using 7.5% polyacrylamide gels (100×120×0.9 $mm^2$). The separating and stacking gels were prepared without SDS, which was added to the running and sample buffers. Samples were heated at 100 °C for 2 min, and electrophoresis was carried out at 15 mA and 7–10 °C. The gels were subsequently processed for Western blot.

### *Western blot*

Blotting was performed according to the protocol described by Towbin et al. (1979). Hemolymph proteins separated by SDS-PAGE were transferred to a PVDF membrane (Millipore, Immobilon-P), which was subsequently incubated in a 1:2000 diluted primary antibody (serum anti-honey bee Vg obtained by immunizing rabbits, Bitondi and Simões, 1996). Membrane- immobilized Vg was detected with Horseradish Peroxidase (HRP) labeled secondary antibody diluted 1:12000 (ECL® kit, Amersham Biosciences). Fluorescent Vg bands were visualized by exposing membranes to a Kodak XR-Omat film. Vg band in stingless bees was identified using the honey bee Vg migrating at the 180 kDa region (Engels et al., 1990; Wheeler and Kawooya, 1990) as a molecular mass marker.

## Results

### *Identity of the cDNA fragments isolated from the stingless bee species*

cDNA fragments were obtained by RT-PCR using total RNA and specific primers for the honey bee *vg* gene. Fragments of about 500 bp were purified, cloned and sequenced. BLASTX analysis of the conceptual trans-

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SpVg        ------------------------------------------------------------
AmVg        RFIGLTSDKFDVSLALDGERVMLKASEDYRYSVRGLCGNFDHDSTNDFVGPKNCLFRKP 1620
FvVg        ----------------------------------------DSTNDFVGPKNCLFRKP
MsVg        ----------------------------------------DSTNDFVGPKNCLFRKP

SpVg        ------------------------------------------------------------
AmVg        EHFVASYALISNQCEGDSLNVAKSLQDHDCIRQERTQQRNVISDSESGRLDTEMSTWGYH 1680
FvVg        EHFVASYALISNQCEGDSLNVAKSLQDHDCIRQERTQQRNVISDSESGRLDTEMSTWGYH
MsVg        EHFVASYALISNQCEGDSLNVAKSLQDHDCIRQERTQQRNVISDSESGRLDTEMSTWGYH

SpVg        --------------------ICFTMRPVVSCASGCTAVETKSKPYKFHCMEKNEAAMKLK
AmVg        HNVNKHCTIHRTQVKETDDKICFTMRPVVSCASGCTAVETKSKPYKFHCMEKNEAAMKLK 1740
FvVg        HNVNKHCMIHRTQVKETDDKICFTMRPVVSCASGCTAVETKSKPYKFHCMEKNEAAMKLK
MsVg        HNVNKHCTIHRTQVKETDDKICFTMRPVVSCASGCTAVETKSKPYKFHCMEKNEAAMKLK

SpVg        KRIEKGANPDLSQKPVSTTEELTVPF----
AmVg        KRIEKGANPDLSQKPVSTTEELTVPFVCKA 1770
FvVg        KRIEKGANP----------------------
MsVg        KRIEKGANPDLSQKPVSTTE----------
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**Figure 1.** Alignment of Vg partial sequences from the stingless bees *S. postica* (SpVg), *F. varia* (FvVg) and *M. scutellaris* (MsVg) with *A. mellifera* (AmVg). Only a fragment of the complete AmVg sequence is shown. The Vg partial sequences in stingless bees showed 100% identity to the corresponding protein from *A. mellifera*. Amino acids in the *A. mellifera* sequence are numbered on the right margin. Letters on a grey background show the motifs GL/ICG (conserved in all insect Vgs), DGXR [that occur in all insect vitellogenins (Tufail et al., 2000) except in the cockroach *L. maderae* (Tufail and Takeda, 2002)], and RXXR/S, a putative cleavage site (Sappington and Raikhel, 1998). Eight from nine of the cysteine residues downstream of GL/ICG were also identified at conserved positions near the C-terminal.

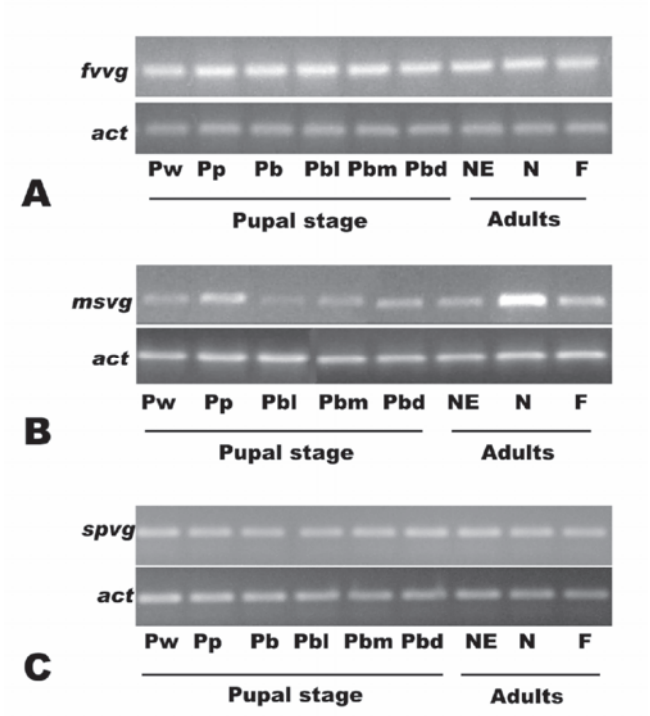


**Figure 2.** Vg mRNA abundance during pupal and adult development of **(A)** *F. varia* (*fvvg*), **(B)** *M. scutellaris* (*msvg*) and **(C)** *Scaptotrigona postica* (*spvg*) females. Semiquantitative RT-PCR followed by electrophoresis of PCR products in ethidium bromide stained agarose gels. An actin gene (*act*) constitutively expressed in *A. mellifera* was used as a control to correct for differences in cDNA amounts. The early (Pw, Pp), intermediary (Pb, Pbl, Pbm), and late (Pbp) phases of pupal stage are indicated. NE, N and F are newly emerged, nurse and forager workers, respectively.

lation products revealed 100% similarity to honey bee Vg. Figure 1 shows the sequence alignments evidencing the presence of conserved motifs and amino acids.

*Developmental patterns of Vg mRNA in the stingless bee workers*

Vg transcripts were detected during the entire pupal development and in adult stages of the three stingless bee species here studied. *F. varia* and *S. postica* showed constant levels of Vg mRNA during the entire pupal development and in adult stages. This contrasts to what was found in honey bees, in which Vg mRNA was not observed in the earlier, but in the later phases of pupal development, reaching the highest level in nurse bees (Piulachs et al., 2003). Differently from *F. varia* and *S. postica*, *M. scutellaris* workers presented variable levels of Vg mRNA during pupal and adult development. Like honey bees, *M. scutellaris* workers showed the highest level of Vg mRNA during nurse stage (Fig. 2).

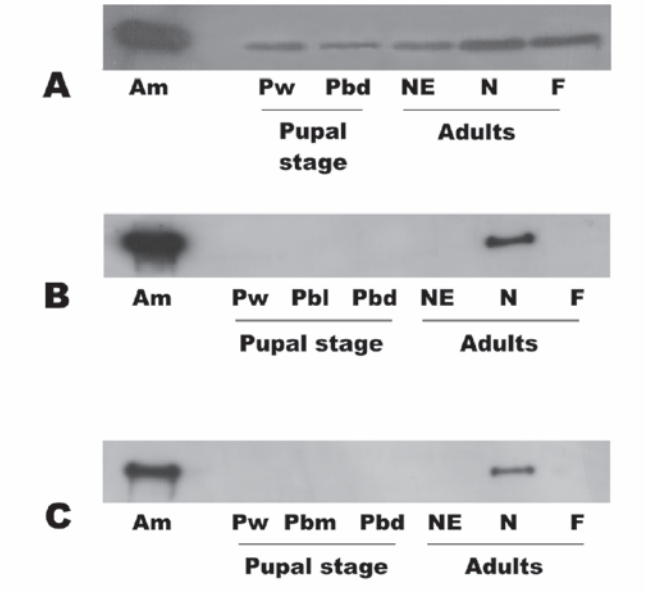


**Figure 3.** Vg protein abundance in hemolymph during pupal and adult development of **(A)** *F. varia*, **(B)** *M. scutellaris*, and **(C)** *S. postica* females. Western blot using anti-serum against honey bee Vg. Vg was identified in nurse bees (N) of the three stingless bees species, and in *F. varia* pupae (Pw and Pbd), newly emerged (NE), nurse (N) and forager (F) workers. A hemolymph sample from *A. mellifera* nurse bees (Am) was used for comparison.

*Developmental patterns of Vg protein in the stingless bee workers*

To investigate the presence of Vg in pupae and adults, we performed Western blots using anti-serum against honey bee Vg. Reactive bands were observed in hemolymph samples from *M. scutellaris* and *S. postica* at the nurse stage. *F. varia*, however, revealed Vg bands during all pupal development and in the adult stages. In this species, nurses and foragers presented higher levels of Vg than pupae and newly emerged adults (Fig. 3).

*Vg mRNA levels in* F. varia *fed or not on dietary proteins –*

The levels of Vg transcript were higher in bees fed on beebread plus sugar than in bees fed only on sugar during 6 or 12 days (from adult emergence). Males presented a similar response to the lack of dietary proteins (Fig. 4). Therefore, like *A. mellifera* workers (Guidugli-Lazzarini, 2006), *F. varia* workers need dietary proteins to increase *vg* transcription. The same was found for *F. varia* males. Figure 4 also shows that, comparatively, 6-day-old females have higher amount of Vg transcript than males of the same age, which received the same diet. At the 12$^{th}$ day, however, similar transcript levels were seen for both sexes. This suggests differences in the rate of *vg* mRNA synthesis and accumulation between sexes.

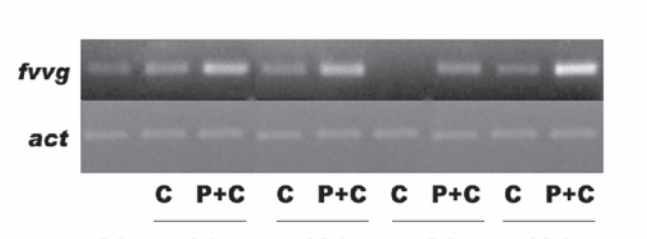


**Figure 4.** Vg mRNA abundance in *F. varia* (*fvvg*) females and males fed on protein (beebread) plus carbohydrate (sugar-cane) (P + C) or only carbohydrate (sugar-cane) (C). Semiquantitative RT-PCR followed by electrophoresis of PCR products on ethidium bromide stained agarose gel. Actin (*act*) was used as housekeeping gene. 6 d, 12 d: time of feeding from emergence to the 6th or 12th day of adult life. 4 d: mRNA from 4-day-old workers, naturally fed in the colony, was used for comparison.

## Discussion

### *Vg expression in egg-laying versus sterile worker bees*

There is little information on Vg in stingless bees. The available data on this subject were recently included in a review (Hartfelder et al., 2006) that emphasized how remarkable stingless bees are in regard to the variability in egg-laying behavior. In this context, the current study adds detailed information on Vg expression in three species of stingless bees differing in respect to egg-laying activity (some of these results were previously mentioned in the above cited review).

We observed that the profile pattern of Vg mRNA in *S. postica* and *M. scutellaris* workers, contrasted with the pattern of Vg accumulation in hemolymph. In both species, Vg mRNA was detected during all the pupal stage and in the newly emerged, nurse and forager workers, whereas the protein was found only in the hemolymph of the nurse bees. This discrepancy could reflect a translational control of Vg biosynthesis, or yet, a post-translational control linked up to Vg secretion and accumulation in hemolymph.

Like *S. postica* and *M. scutellaris*, the permanently sterile *F. varia* workers showed Vg mRNA during all pupal and adult stages. But only in the latter, Vg protein was detected through all these stages. Therefore, in *F. varia* workers, developmental patterns of Vg mRNA matched the respective protein patterns. Particularly in this case, Vg production is totally dissociated from egg-laying activity, since these workers do not produce eggs. Even so, like the stingless bee workers that usually lay eggs (*M. scutelaris* and *S. postica*) maximal levels of Vg were found in *F. varia* in the nurse stage. Thus, independently of the reproductive potential, Vg accumulates in the hemolymph of young stingless bee workers.

The detection of Vg in the functionally sterile *A. mellifera* workers, and also in drones, although in small amounts (Trenczek et al., 1989), first motivated the statement that Vg could have an alternative function besides being a yolk component. Engels et al. (1990) suggested a function in lipid transport. Later, Amdam and Omholt (2002) and Amdam et al. (2003, 2004) proposed its participation in the synthesis of brood food, transport of zinc, longevity, and immune response. More recently, RNAi experiments pointed to a function of Vg in regulating the hemolymphatic titer of juvenile hormone (Guidugli et al., 2005a). The presence of Vg in the permanently sterile *F. varia* workers strongly reinforces that Vg has other important functions in bee physiology, besides its fundamental role in vitellogenesis.

As observed in *Apis mellifera* (Guidugli-Lazzarini, 2006), the levels of Vg mRNA in *F. varia* workers (the current study) were strictly correlated to ingestion of pollen, a rich protein source. Even in *F. varia* males, the abundance of Vg mRNA was affected by the quality of the diet. It is known that the progressive increase in total protein content in hemolymph of young *A. mellifera* workers occurs at the expenses of Vg secretion during the initial period of the adult life cycle, when workers intensively consume pollen (Bitondi and Simões, 1996). In the same way as honey bees, the increase in the hemolymphatic stock of Vg in the stingless workers depends on pollen consumption. Thus, a nutritional control equivalent to that occurring in honey bee workers, which may use Vg for egg development, was observed in workers of a stingless bee species, which will never use Vg for this purpose.

At the adult life cycle, when honey bees substitute pollen- for nectar-consumption, the levels of Vg go into a progressive decline (Engels et al., 1990). The physiological adjustment resulting from this radical modification in the diet is associated with a decisive switch in the task-performing schedule. Thus, honey bee workers that were exclusively working inside the nest start foraging for nectar and pollen. In *S. postica* and *M. scutellaris* foragers, we also detected a drastic decrease in the hemolymphatic stock of Vg. However, in contrast to these species, Vg was still present in considerable amounts in *F. varia* that were foraging for pollen. A greater plasticity in age-related task-performance was found for *S. postica* (Bego, 1982) and *M. quadrifasciata* (Waldschmidt and Campos, 1997) in comparison to *A. mellifera*, and this perhaps could explain the presence of high levels of Vg in *F. varia* foragers. Comparative studies including workers of known age and task, from different stingless bee species, may further improve our knowledge on task-performing plasticity in the context of bee physiology and activation/repression of gene expression.

Being a storage protein, Vg possibly serves as a source of amino acids to support adulthood of functionally or permanently sterile workers. At the forager stage, when bees ingest almost exclusively carbohydrates, the hemolymphatic stock of Vg is gradually depleted, possibly to attend for amino acid needs. At the end of the adult life cycle, the drastic depletion of Vg stock in honey bee foragers apparently is the direct cause of reduction in the number of hemocytes, down-regulation of cell-defense machinery, and consequent senescence and death

(Amdam et al., 2004). This scheme may also be valid for stingless bees, but given their plasticity in age-related task performance (which certainly implies in a considerable flexibility in the associated physiological processes), distinct dynamics of Vg accumulation/depletion may occur in these bees.

### *Pupal pattern of Vg expression in stingless bee workers and ecdysteroid titers*

In *A. mellifera* pupae, the onset of Vg expression is negatively regulated by ecdysteroids. Vg (mRNA and protein) only appears in hemolymph at the end of the pupal stage, after ecdysteroid titer has decayed (Barchuk et al., 2002; Guidugli et al., 2005b). With minimal specie-specific variations, ecdysteroid levels in stingless bees (as determined for *S. postica* and *M. quadrifasciata*) (Hartfelder and Rembold, 1991; Pinto et al., 2002) follow the same profile pattern described for *A. mellifera*, with an increase during the middle of the pupal stage followed by a decrease at the end of this stage. In *M. scutellaris*, the lower levels of Vg mRNA transcript during the middle of pupal stage, when maximal levels of ecdysteroid should take place, evoke the negative control described for *A. mellifera*. In the Trigonini species here studied, on the contrary, the invariable abundance of Vg mRNA during pupal stage rejects any proposal of negative regulation by ecdysteroids. It was suggestive that *M. scutellaris*, which in the phylogenetic scale is closer to honey bees than *S. postica* and *F. varia*, has shown a Vg mRNA pattern that better approximates that described for *A. mellifera*.

In conclusion, distinct patterns of Vg expression were observed among the stingless bee species here studied. Interestingly, Vg protein was found to be constitutively present in *F. varia* workers, which never lay eggs. The fact that stingless bee workers produce Vg, independently of their participation in egg-laying activity, is consistent with a role of this protein in essential aspects of honey bee physiology, besides yolk formation for egg development.

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