Archives of Insect Biochemistry and Physiology 59:211–218 (2005)

Vitellogenin Expression in Queen Ovaries and in Larvae of Both Sexes of *Apis mellifera*

Karina R. Guidugli,[1] Maria-Dolors Piulachs,[2*] Xavier Bellés,[2] Anete P. Lourenço,[1] and Zilá L.P. Simões[3*]

In the honeybee, *Apis mellifera*, vitellogenin (Vg) expression has been detected in the ovary of queens, but not in that of workers. In addition, larvae of both sexes produce Vg in significant amounts, which suggest that Vg serves for functions additional to oocyte growth and energy supply to the embryo. In vivo hormone treatment experiments suggest that the decrease of 20-hydroxyecdysone concentration occurring in previtellogenic phases allows Vg production. Southern analysis indicates that the Vg gene is present as a single copy in the honeybee genome. Arch. Insect Biochem. Physiol. 59:211–218, 2005.

KEYWORDS: vitellogenin; *Apis mellifera*; honeybee; 20-hydroxyecdysone; juvenile hormone

INTRODUCTION

In insects, vitellogenin (Vg), the precursor of the major yolk protein, vitellin, is synthesized by the fat body, released to the hemolymph, and afterwards internalized in developing oocytes, where it serves as a source of energy for the embryo (Wyatt, 1999; Raikhel et al., 2004). However, there are species in which Vg is also produced by the ovaries, or by female immature stages, or even by males (Valle, 1993; Satyanarayana et al., 1994; Yano et al., 1994; Bellés, 2004; Raikhel et al., 2004). This suggests that Vg may have other functions in addition to oocyte maturation and energy supply for embryogenesis.

In *Apis mellifera*, Vg has been studied mainly in the context of oocyte maturation (Engels et al., 1990; Hartfelder and Engels, 1998; Barchuk et al., 2002; Piulachs et al., 2003). In freshly emerged queens, Vg begins to accumulate in the hemolymph, and within only 3 days it represents up to 70% of the hemolymph proteins (Engels et al., 1990; Hartfelder and Engels, 1998), and this high production is maintained throughout adult life. However, even though considered functionally sterile, workers also synthesize Vg (Engels et al., 1990), and in a typical colony of *A. mellifera*, during the first 7 days of a worker's adult life (when it becomes a nurse bee and performs brood-rearing tasks), Vg titer increases and may account for up to 40% of the total hemolymph proteins (Engels et al., 1990). Moreover, trace amounts or even significant quantities of Vg are found in adult males (Trenczek and Engels, 1986; Trenczek et al., 1989; Valle, 1993; Piulachs et al., 2003). Data on workers and drones point to Vg functions related to me-

[1]Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto-SP, Brasil

[2]Department of Physiology and Molecular Biodiversity, Institut de Biologia Molecular de Barcelona (CSIC), Barcelona, Spain

[3]Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto-SP, Brasil

Contract grant sponsor: Fundação de Amparo à Pesquisa do Estado de São Paulo, Brasil; Contract grant number: FAPESP 99/00719-6; Contract grant sponsor: Ministry of Education and Science, Spain; Contract grant numbers: BOS2002-03359 and AGL2002-01169.

*Correspondence to: Maria-Dolors Piulachs, Departament of Physiology and Molecular Biodiversity, Institut de Biologia Molecular de Barcelona (CSIC), Jordi Girona 18, 08034 Barcelona, Spain. E-mail: mdpagr@cid.csic.es; or Zilá L.P. Simões, Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto-SP, Brasil. E-mail: zlpsimoe@usp.br

Received 11 January 2005; Accepted 2 March 2005

DOI: 10.1002/arch.20061
Published online in Wiley InterScience (www.interscience.wiley.com)

tabolism, as postulated by Amdam and Omholt (2002).

Taking advantage of the recent cloning of Vg cDNA in *A. mellifera* (Piulachs et al., 2003), the present work studies the expression of Vg in terms of mRNA in the ovaries of queen and worker, and in immature stages of all castes and both sexes. The idea is to illuminate the spectrum of sources and functions of honeybee Vg.

MATERIALS AND METHODS

Insects

Specimens of Africanized *A. mellifera* were obtained from hives of the experimental apiary of the Department of Genetics, Faculty of Medicine in Ribeirão Preto, University of São Paulo, Brazil. Queen, worker, and drone larvae and adults were removed from brood frames and maintained in an incubator at 34°C and 80% r.h., that is, in conditions similar to those of the colony. Pupae were staged using eye color and intensity of cuticle pigmentation as criteria, according to Michellete and Soares (1993) and Barchuk et al. (2002). Larvae age was assessed by a relative time schedule based on morphological criteria (Rachinsky et al., 1990).

Western Blot Analysis

Soluble hemolymph proteins (1–10 μg) were separated by SDS-PAGE on 7.5% acrylamide gels, transferred to a polyvinylidene fluoride membrane (Millipore, Immobilon 0.45 μ), and processed for Western blot. Membranes were treated with an antiserum against honeybee egg proteins raised on rabbit (Bitondi and Simões, 1996), used at a dilution of 1:500, and incubated with a peroxidase-anti peroxidase system (DAKO, Denmark, Z 113), using diaminobenzidine (Vector SK-4100) as chromogen (Bitondi and Simões, 1996). Protein concentration was determined according to Bradford (1976).

Northern Blot Analysis

In larvae and pupae, total RNA was extracted from the entire body and the abdomen, respectively. In adults, abdominal fat bodies were studied. For that purpose, the gut, the accessory glands, and the ovaries were removed and the abdominal carcass containing the fat body was processed for RNA extraction. One entire larval body, pupal abdomen, or two adult abdominal carcasses with the corresponding fat body were extracted with 1 ml of TRIzol reagent (Invitrogen, La Jolla, CA) following the manufacturer's instructions. To study Vg expression in ovaries, total RNA was extracted from pools of 2–4 ovaries from freshly emerged queens, or pools of 20–40 ovaries from 10- to 25-day-old workers. Total RNA was subjected to electrophoresis in 1.2% agarose gel containing formaldehyde and then transferred to nylon membranes (Hybond-N^+, Amersham Biosciences, Arlington Heights, IL). The probe used to detect Vg corresponds to the fragment between nucleotides 4809–5540 of *A. mellifera* Vg cDNA (sequence accession number AJ517411). The probe was labeled with fluorescein using the Gene Image labeling kit (Amersham Biosciences). Hybridization and detection procedures were as described by Martín et al. (1998), using the Gene Image CDP-star detection module (Amersham Biosciences).

Estimation of Vitellogenin mRNA Levels With RT-PCR and Southern Blot

One microgram of each RNA preparation was DNAse treated (Promega, Madison, WI) and reverse transcribed with Superscript II reverse transcriptase (Invitrogen) and Oligo $(dT)_{12\text{-}18}$ (Invitrogen). For Vg detection, these cDNA samples were subjected to PCR amplification with 25 cycles at 94°C (30 s), 62°C (30 s), and 72°C (1 min). The same cDNA samples were subjected to 20–25 cycles of PCR with specific primers for the ribosomal 28S subunit or for the actin transcript. Southern blot probes were generated by PCR with the same primer pairs using cDNA as a template. The primers for Vg were: forward, 5′ ACG ACT CGA CCA ACG ACT T 3′, and reverse, 5′ AAC GAA AGG AAC GGT CAA TTC C 3′; for 28S: forward, 5′ AAT CCT CGT GGT TCA TCG G 3′, and reverse, 5′ TTC TCC AGC TCA GTC CTC C 3′; and for actin: forward, 5′ TGC CAA CAC TGT CCT TTC TG 3′, and reverse,

5′ AGA ATT GAC CCA CCA ATC CA 3′. RT-PCR followed by Southern blotting of total RNA without reverse transcription did not result in amplification, indicating that there was no genomic contamination. Labeling of probes and detection were as described above.

Hormone Treatment

Brown-eyed worker pupae (Pb) were carefully collected from combs and divided into the following five groups containing 30 bees each: (1) untreated, (2) treated with the JH analogue pyriproxyfen (2-[1-methyl-2(4-phenoxyphenoxy) ethoxyl] pyridine (from Sumitomo), (3) treated with 20-hydroxyecdysone (20E) (from Fluka Chemie, Buchs, Switzerland), (4) treated with acetone (control of pyriproxyfen), and (5) treated with ethanol diluted in Ringer (control of 20E). Treatment of Pb pupae was performed before the increase of ecdysteroids in the hemolymph (Hartfelder and Engels, 1998). Pb pupae were topically treated with 1 µg of pyriproxyfen in 1 µl acetone, or injected with 5 µg of 20E in 1 µl of ethanol/Ringer (1:3 v/v) solution. Bees were maintained in an incubator at 34°C and 80% r.h. for 72 h, when untreated bees were in Pbd stage (brown-eyed pupae and dark pigmented cuticle) and started Vg synthesis (Barchuk et al., 2002; Piulachs et al., 2003). At this time, samples (n = 2) of each group were collected, and total RNA was extracted. Two or three entire pupal abdomens were extracted in 1 ml of TRIzol reagent (Invitrogen) following the manufacturer's instructions.

Determination of Vitellogenin Gene Copies by Southern Blot

For DNA extraction, 10 freshly molted pupae were snap-frozen in liquid nitrogen and ground to a fine powder. Subsequently, the macerated material (100 mg) was transferred to 2-ml polypropylene tubes containing 500 µl of extraction buffer (0.1M NaCl, 1 mM EDTA, 1.5% SDS, 50 mM Tris-HCl, adjusted to pH 7.5) plus proteinase K (0.5 mg ml^{-1}). After incubation at 55°C for 2 h, the homogenate was centrifuged (12,000*g* for 10 min) and DNA was extracted from the supernatant by repeated phenol-chloroform liquid-phase separation, followed by isopropanol precipitation. Samples of *A. mellifera* genomic DNA (20–30 µg) were treated with RNase, cleaved with *Bam*HI and *Eco*RI and run on a 1% agarose gel to obtain a Southern blot. Procedures for labeling and detection of Vg were as described for Northern blot.

RESULTS AND DISCUSSION

Vitellogenin Expression in the Ovary

Cytological studies on the ovaries of larvae and adults of *A. mellifera* queens (Fleig, 1995) had suggested that the follicular cells of the queen ovary produce Vg. To assess this possibility, we studied the expression of Vg in the ovary of *A. mellifera* queens. Northern blot analysis revealed that adult queens express Vg in the ovary, although at lower levels compared with the fat body (Fig. 1). In contrast, we were unable to detect Vg mRNA in ovarian tissue of workers (Fig. 1). This result was confirmed by RT-PCR using mRNA from ovaries of 9-, 12-, and 25- to 30-day-old workers (data not shown), indicating that ovarian Vg expression in

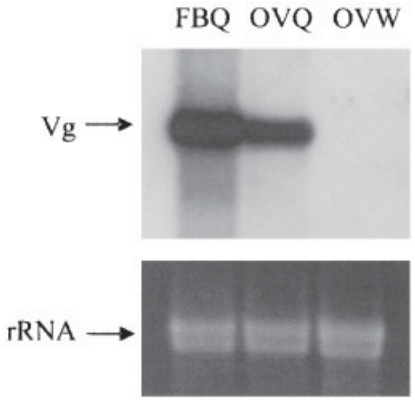

Fig. 1. Expression of vitellogenin mRNA in *A. mellifera* ovaries. Northern blot of total RNA from fat body of newly emerged queens (FBQ; 3 µg); from ovaries of newly emerged queens (OVQ; 4.5 µg); and from the ovaries of 10- to 25-day-old workers (OVW; 12 µg). The portion of the gel containing rRNA was stained with ethidium bromide as a control of sample loading. The image is representative of two replicates.

A. mellifera is queen specific. Although the fat body has been considered as the exclusive site of Vg synthesis in the majority of insects (Bellés, 2004; Raikhel et al., 2004), ovarian Vg production has been reported in a number of species. In Diptera, production of yolk proteins in follicle cells is quite common and has been reported in *Drosophila melanogaster, Musca domestica, Calliphora vicina, C. erythrocephala, Sarcophaga bullata, Dacus oleae, Anastrepha suspensa*, and *Stomoxys calcitrans* (Bellés, 2004; Raikhel et al., 2004). In non-dipteran insects, ovarian production of Vg has been described only in the firebrat *Thermobia domestica* (Rousset and Bitsch, 1993), the beetles *Coccinella septempuctata* (Zhai et al., 1984) and *Leptinotarsa decemlineata* (Peferoen and DeLoof, 1986), and the bug *Rhodnius prolixus* (Melo et al., 2000). However, the list could surely be enlarged after assessing the vitellogenic role of the ovary in a larger sample of species, especially in those having meroistic ovaries.

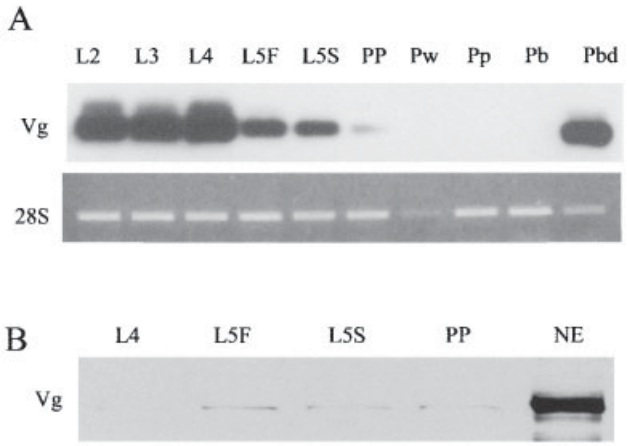

Fig. 2. Vitellogenin expression during preimaginal development of *A. mellifera* workers. **A:** Semiquantitative RT-PCR analysis of vitellogenin (Vg). Amplification of 28S transcript was used as control of sample loading. L2, L3, L4, L5F, and L5S = larval stage; PP = prepupae; Pw, Pp, Pb, Pbd = pupal phase, according to Michellete and Soares (1993). **B:** Western blot analysis of hemolymph Vg in preimaginal development of *A. mellifera* workers. SDS-PAGE (7.5%) was performed with 10 μg of total proteins from larvae (L4, L5F, and L5S), or prepupae (PP) hemolymph. One microgram of proteins from hemolymph of new emerged (NE) workers was used as a positive control. Images are representative of two replicates.

Vitellogenin Expression in Larvae

Production of Vg is often related to oocyte maturation and studies are mainly based on the adult stage. However, in *A. mellifera* Vg expression has been detected not only in adults, but also in late pupae and pharate adults of all castes (Engels et al., 1990; Hartfelder and Engels, 1998; Barchuk et al., 2002; Piulachs et al., 2003). Thus, we were interested in studying whether Vg is expressed in younger stages of different castes, using total RNA and protein extracts, and RT-PCR analysis and Western blot. In worker larvae, and in terms of mRNA, Vg expression was detected at high levels early in development (second, third, and fourth larval instars), then expression decreased in fifth (last) larval instar and in prepupae, became practically undetectable during the early pupae (Fig. 2A), and increased again in late pupae (Fig. 2A), showing levels similar to those reported in young adults by Piulachs et al. (2003). In terms of protein, Vg was detected in the last larval and prepupal stages at very low levels, compared with those observed in newly emerged specimens (Fig. 2B), but not in early pupae (not shown). Moreover, the comparison of mRNA and Vg data indicate that there is no clear correspondence between both parameters in pre-adult stages (compare Fig. 2A and 2B). In fourth instar larvae, for example, high mRNA levels correspond to very low Vg amounts, which suggests the occurrence of mechanisms operating on the translatability and/or stability of Vg mRNA. Equivalent expression studies performed on queen and drone larvae indicated that the pattern of Vg mRNA is similar in both sexes and in both castes of *A. mellifera*, showing high levels in larvae and decreasing towards the prepupal stage (Fig. 3). Synthesis of Vg during the larval period, not only in females (queens and workers) but also in males (drones), is atypical in insects, and suggests that Vg may have functions other than serving as yolk precursor in *A. mellifera*. Recent studies have indicated that insect Vg may be involved in the transport of sugars, lipids, phosphates, vitamins, and

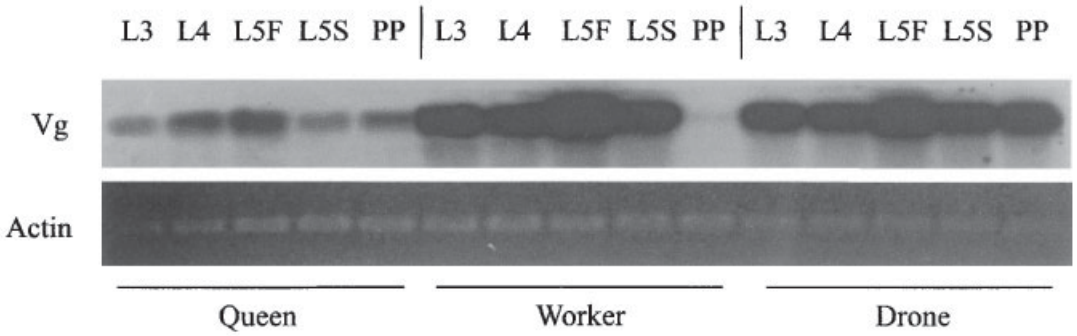

Fig. 3. Semiquantitative RT-PCR analysis of vitellogenin (Vg) expressed during preimaginal development in different castes of *A. mellifera*. Amplification of actin transcript was used as a control of sample loading. L3, L4, L5F, and L5S = the larval stage; PP = prepupae, according to Michellete and Soares (1993). Film was overexposed to see the amplification in PP workers. The image is representative of two replicates.

hormones (Chen et al, 1997; Sappington and Raikhel, 1998). In addition, Vg in *A. mellifera* has been implicated in metabolic functions like the synthesis of brood food, transport of zinc, longevity, and immune system (Amdam and Omholt, 2002; Amdam et al., 2003, 2004). These possible functions may explain the patterns observed in our studies.

Effects of Juvenile Hormone Analogue and 20-Hydroxyecdysone

Our results indicate that Vg expression follows characteristic developmental patterns that point to underlying endocrine control mechanisms. Rachinsky et al. (1990) have reported the ecdysteroid and JH titers in *A. mellifera* workers. Ecdysteroids are generally low in last larval instars, increase notably in the prepupae and the pupae, and decrease towards late pupae and in the adult (Rachinsky et al., 1990; Pinto et al., 2002). In contrast, JH levels are generally low in the last larval instar, prepupae, and pupae, and slightly increase in the pharate adult (Rachinsky et al., 1990). In line with these patterns, Barchuk et al. (2002) proposed that one of the determinants of Vg synthesis in *A. mellifera* is the decrease of ecdysteroid levels. We treated worker pupae in Pb stage (that is, just before the increase of ecdysteroid titer), with the JH analog pyriproxyfen or 20E, and Vg expression was analyzed 72 h later. The treatment with 20E reduced Vg expression, whereas that of pyriproxyfen was practically ineffective (Fig. 4). These results are consistent with hormonal levels reported by Rachinsky et al. (1990) and with the hypothesis proposed by Barchuk et al. (2002).

Vitellogenin Gene Copies

Multiple Vg gene copies have been demonstrated in the insects *Locusta migratoria* (Dhadialla et al., 1987), *Aedes aegypti* (Chen et al., 1994; Romans et al., 1995), *Riptortus clavatus* (Hirai et al., 1998), *Plautia stali* (Lee et al., 2000), and *Periplaneta*

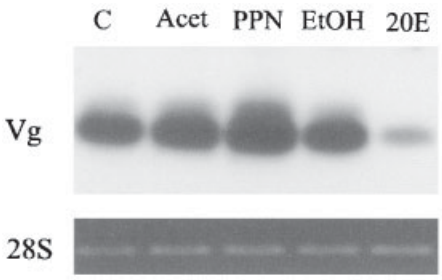

Fig. 4. Vitellogenin (Vg) expression in *A. mellifera* workers after hormonal treatment. Workers were treated in Pb stage and analyzed 72 h later. C: Untreated control; Acet: Acetone control (of pyriproxyfen); PPN: treated with pyriproxyfen; EtOH: Ethanol/Ringer control (of 20-hydroxyecdysone); 20-E: treated with 20-hydroxyecdysone. Amplification of 28S transcript was used as a control of sample loading. The image is representative of two replicates.

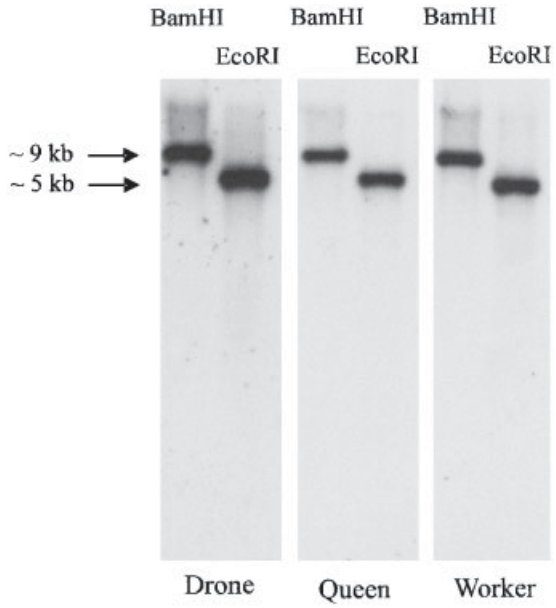

Fig. 5. Southern blot analysis from *A. mellifera* genomic DNA. Genomic DNA extracted from unpigmented pupae of queen, worker, and drone was single-digested with *Eco*RI and *Bam*HI, separated and blotted, and then was probed with Vg cDNA. The molecular size (kb) of each fragment is shown on the left.

americana (Tufail et al., 2001). It has been suggested that multiple genes may be necessary in species needing to provide a huge amount of Vg to grow oocytes in a relatively short period of time (Tufail et al., 2001). Results obtained herein suggesting that Vg could have more general metabolic functions in all castes of *A. mellifera* point to the possibility that the Vg gene is represented by multiple copies. Therefore, a genomic Southern analysis was done using a cDNA fragment as a probe to determine the number of Vg gene copies in queen, workers (diploid genome), and drones (haploid genome) of *A. mellifera*. The probe hybridized to a single genomic fragment in all cases (Fig. 5). These results indicate that there is a single copy of Vg in the *A. mellifera* haploid/diploid genome. This is consistent with the finding of a unique sequence in the honeybee genome sequencing project (gnl|Amel_1.1|Contig 2354; Donnell, 2004), after launching BLAST with vitellogenin cDNA as a virtual probe. The presence of a single copy of the Vg gene has been reported in a number of insect species, like *Lymantria dispar* (Hiremath and Lethoma, 1997), *Bombyx mori* (Yano et al., 1994), *Manduca sexta* (Adamczyk et al., 1996), *Athalia rosae* (Matsumoto et al., 2002), and *Encarsia formosa* (Donnell, 2004).

ACKNOWLEDGMENTS

The authors thank Luís Roberto Aguiar for technical assistance in the apiary and Aline dos Santos Market and Alexandre dos Santos Cristino for their help in the experimental procedures. K.R.G. was supported by a fellowship from FAPESP.

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