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The vitellogenin of the honey bee, *Apis mellifera*: structural analysis of the cDNA and expression studies

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Abstract

The cDNA of *Apis mellifera* vitellogenin was cloned and sequenced. It is 5440 bp long and contains an ORF of 1770 amino acids (including a putative signal peptide of 16 residues). The deduced amino acid sequence shows significant similarity with other hymenopteran vitellogenins (58% with *Pimpla nipponica* and 54% with *Athalia rosae*). The alignment with 19 insect vitellogenins shows a high number of conserved motifs; for example, close to the C-terminus there is a GL/ICG motif followed by nine cysteines, as occurs in all hymenopteran species, and, as in other insect vitellogenins, a DGXR motif is located 18 residues upstream the GL/ICG motif. Phylogenetic analysis of vitellogenin sequences available in insects gave a tree that is congruent with the currently accepted insect phylogenetic schemes. Using two fragments of the vitellogenin cDNA as probes, we analyzed by Northern blot the sex- and caste-specific patterns of vitellogenin expression in pupae and adults of *A. mellifera*. In queens, vitellogenin mRNA was first detected in mid-late pupal stage, whereas in workers it was first detected in late pupal stage. Vitellogenin mRNA was also observed in drones, although it was first detected not in pupae but in freshly molted adults.
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1. Introduction

Reproduction is especially interesting in social insects due to the occurrence of castes, that is, the presence of a highly fertile queen contrasting with more or less sterile workers. In most species of social bees, each female larva has the potential to become a queen if it is appropriately fed. In other words, queens and workers are genetically equivalent. To become a queen, a larva must receive more food, or be fed with a special diet for a longer period of time, than larvae developing into workers. These differences in diet elicit an endocrine response that induces a caste-specific developmental program.

In the honey bee, this sequence of events has been studied in detail in connection with the development of the ovaries. A high juvenile hormone titer during most of the larval period (Rachinsky et al., 1990) guarantees the survival of practically all the ovariole anlagen to the adult stage, with the result that each ovary of an adult queen contains up to 180 ovarioles. Therefore, the queen can lay more than 1000 eggs per day. In the worker, in contrast, only a few (2–12) of the ovariole anlagen survive metamorphosis (Hartfelder and Steinbrück, 1997; Schmidt-Capella and Hartfelder, 1998).

Another important difference between castes related to reproduction is the earlier onset of vitellogenin synthesis in queens, which starts 60 h before adult emergence. Shortly after emergence, vitellogenin begins to accumulate in the hemolymph, and within only three days it represents up to 70% of the queen's hemolymph proteins (Barchuk et al., 2002). This high production is maintained throughout adult life. Even though considered functionally sterile, workers also synthesize vit-

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ellogenin (Engels et al., 1990). Appearance of vitellogenin in workers starts later than in the queen, beginning approximately 10 h before emergence. Thereafter, vitellogenin can still be detected in the hemolymph, although at clearly lower levels than in the queen (Barchuk et al., 2002).

During the first 7 days of the worker adult life, when it becomes a nurse bee and performs brood-rearing tasks, the vitellogenin titer increases and may account for up to 40% of the total hemolymph proteins (Engels et al., 1990). The small, slender ovaries contain between 2 and 12 ovarioles each, although they are not activated. In the presence of an egg-laying queen, the workers of the colony perform a series of age-related tasks, termed age polyethism, and when they reach the age of 15 days they undergo a series of changes in their behavior and physiology, the most remarkable of which is an increase in the juvenile hormone titer (Robinson, 1992). Their salivary glands then begin to degenerate, especially the hypopharyngeal branches, and they switch from a protein-rich (pollen) to a carbohydrate-rich (honey and nectar) diet as they become foragers and will collect nectar, pollen and water (Bitondi and Simões, 1994, 1996; Crailsheim and Stolberg, 1989). If a colony loses its queen, this characteristic sequence in task performance is largely lost. In these conditions, some of the younger workers show signs of ovary activation and significantly increase the vitellogenin titer (Simões, unpublished data). Since they are unable to mate, their eggs remain unfertilized and give rise to drones.

Vitellogenin is generally considered a female-specific protein, which is generally synthesized by the fat body (Bellés, 2003; Wyatt and Davey, 1996). In social insects, such as the honey bee, the presence of high vitellogenin levels in the hemolymph of reproductively active females, such as the queen, is not surprising. However, low vitellogenin levels in the hemolymph of workers, which could activate the ovaries only exceptionally, and the fact that vitellogenin can also be detected in the hemolymph of drones (Barchuk et al., 2002; Trenczek et al., 1989), make these insects an interesting model to study the regulation of vitellogenin expression at molecular scale. To this end, we describe the isolation and sequence of a vitellogenin cDNA of the honey bee, and the use of probes derived from this sequence in Northern blot analysis to assess the developmental and sex specificity of vitellogenin expression.

2. Materials and methods

2.1. Insects

The specimens of Africanized *Apis mellifera* used were from hives of the Experimental Apiary of the Department of Genetics, Faculty of Medicine in Ribeirão Preto, University of São Paulo, Brazil. To obtain pupae of uniform age, queens were periodically confined for 6 h on combs without brood, where they laid eggs. Worker and drone pupae were removed from the brood frames and maintained in an incubator (34 °C and 80% relative humidity). To obtain queen pupae, first instar larvae were transferred to queen cells (with a drop of royal jelly) and reared in a queenless colony until they reached the pupal stage. Pupae were staged according to Michelette and Soares (1993) and Barchuk et al. (2002), using eye color and intensity of cuticle pigmentation as criteria (Table 1). To obtain adult workers of known ages, combs containing workers ready to emerge were removed from colonies and placed in an incubator at 34 °C and 80% relative humidity, conditions that were similar to those of the colony. The workers emerging within 15–20 h were collected and marked on the thorax with paint and placed into a small hive formed by a queen, approximately 3000 bees, brood, honey and pollen.

2.2. Amplification of vitellogenin cDNA

Poly(A)$^+$ RNA was extracted from fat bodies of 2-day-old queens using Straight A's mRNA isolation system from Novagen. A total of 1 μg of Poly(A)$^+$ RNA was used to construct an adaptor-ligated double stranded cDNA library using a Marathon cDNA amplification kit (Clontech). This library was used for the extension of the 3′ end of *A. mellifera* vitellogenin cDNA, as described by Lee et al. (2000a). To amplify the 3′ end of vitellogenin cDNA, two degenerate primers corresponding to the GL/ICG conserved motif were designed (Isof-vitellogenin-1: 5′GGI CT(GC) TG(CT) GG3′; Isof-vitellogenin-2: 5′GGI AT(CT) TG(CT) GG3′), where I corresponds to inosine. The adaptor-ligated double stranded cDNA library was subjected to PCR with Isof-vitellogenin-1 and -2 as forward primers and the

Table 1
Abbreviations of pupal stages, characteristics and time to eclosion, in hours

Hours	Stages	Characteristics
190	Pw	White-eyed pupae, unpigmented cuticle
170	Pp	Pink-eyed pupae, unpigmented cuticle
160	Pb	Brown-eyed pupae, unpigmented cuticle
85	Pbl[a]	Brown-eyed pupae, light pigmented cuticle
60	Pbm[b]	Brown-eyed pupae, intermediary pigmented cuticle
30	Pbd	Brown-eyed pupae, dark pigmented cuticle
10	Pha	Pharate adult (pupae that look like adults)

Modified from Michelette and Soares (1993) and Barchuk et al. (2002).

[a] Pbl was subdivided into I and II according to the progressing cuticle pigmentation.

[b] Pbm was subdivided into I, II and III according to the progressing cuticle pigmentation.

adaptor primer (Marathon cDNA adaptor, Clontech) as reverse, following the supplier's protocol. PCR conditions were: 94 °C for 2 min; 30 amplification cycles of 94 °C for 30 s; 55 °C for 1 min and 72 °C for 2 min, and an extension at 72 °C for 10 min. The Taq polymerase used was from Roche. In order to complete the 5′ end we followed the 'rapid amplification of cDNA ends' (RACE) method (Frohman et al., 1988) using the Invitrogen 5′RACE kit. Oligonucleotides to be used as primers were designed on the basis of the 5′ end of the partial sequence obtained by 3′ end amplification. In order to assess that all amplifications obtained with RACE correspond to the same molecule, the entire cDNA was amplified with a forward primer designed at the 5′ end and a reverse primer designed at the 3′ end just before the Poly(A)$^+$ tail.

2.3. cDNA cloning and sequencing

PCR products were subjected to electrophoresis on 1.2% agarose gel, purified using QIAquick PCR purification kit (Qiagen) and subcloned in pSTBlue-1 Acceptor vector (Novagen). Sequence analysis was performed by dideoxynucleotide chain termination method. Clones were sequenced on both strands using the SP6 and T7 sequencing primers and internal, specific primers, in an automated fluorescence sequencing system ABI (Perkin–Elmer)

2.4. Sequence comparison and analysis

Vitellogenin amino acid sequences used for comparison were from the following species (reference and accession number in parenthesis). The dictyopterans *Blattella germanica* (Comas et al., 2000; AJ005115), *Leucophaea maderae* (Tufail and Takeda, 2002; AB052640) and *Periplaneta americana* (Tufail et al., 2000, 2001; vitellogenin1: AB034804, vitellogenin2: AB047401); the hemipterans *Riptortus clavatus* (Hirai et al., 1998; U97277), *Graptopsaltria nigrofuscata* (Lee et al., 2000b; AB026848) and *Plautia stali* (Lee et al., 2000a; vitellogenin1: AB033498, vitellogenin2: AB033499, vitellogenin3: AB033500); the coleopteran *Anthonomus grandis* (Trewitt et al., 1992; M72980); the hymenopterans *Athalia rosae* (Nose et al., 1997; AB007850) and *Pimpla nipponica* (Nose et al., 1997; AF026789); the lepidopterans *Bombyx mori* (Yano et al., 1994a,b; D13160), *Lymantria dispar* (Hiremath and Lehtoma, 1997a,b; V60186), *Samia cynthia* (AB055844), *Antheraea yamamai* (AB055843) and *Antheraea pernyi* (AB049631); and the dipterans *Aedes aegypti* (Chen et al., 1994; U02548) and *Anopheles gambiae* (AF281078). The software package of the Genetics Computer Group (GCG, version 10.2) of the University of Wisconsin (Devereux et al., 1984) was used for sequences alignments, which were carried out with PILEUP and were not further hand-refined. The percentage of similarity between sequences was estimated using the corresponding application of the Multiple Sequence Alignment Editor, GeneDoc version 2.6.002. Phylogenetic analysis was carried out using amino acid sequences, with the Phylogeny Inference Package (PHYLIP, version 3.57c). We followed the method of Neighbor-Joining, and the distances between different vitellogenins were estimated with Protdist method. Bootstrap analyses were carried out with the application Seqboot in the PHYLIP package, and the procedure was repeated 100 times.

2.5. Northern blot analysis

In pupae, total RNA was extracted from entire abdomens. In adults, abdominal fat bodies were dissected by removing the gut, the accessory glands and the ovaries from the abdomen. Then, the abdominal carcass with attached fat body was processed for RNA extraction. One entire pupal abdomen, or two adult abdominal carcasses with the corresponding fat body, were extracted with 1 ml of TRIzol reagent (Invitrogen) following the manufacturer instructions. Total RNA was subjected to electrophoresis in 1.2% agarose gel containing formaldehyde and then transferred to nylon membranes (Hybond-N$^+$, Amersham Biosciences). Two alternative probes were used on basis of short fragments of *A. mellifera* cDNA, one between nucleotides 3605 and 4905, and the other at position 4809–5540 (see the sequence at database, accession number AJ517411). Fluorescein labeling of the probes was done using Gene image labeling kit (Amersham Biosciences). Hybridization and detection procedures were as described by Martín et al. (1998) using Gene image CDP-star detection module (Amersham Biosciences).

3. Results and discussion

3.1. The vitellogenin cDNA of A. mellifera

The vitellogenin cDNA of *A. mellifera* was cloned following Lee et al. (2000a), using a degenerate primer designed against the GL/ICG motif, which is conserved among all insect vitellogenins, and an adaptor primer at 3′ region. The sequence obtained had 631 bp, corresponding to 3′ end of vitellogenin with a termination codon (TAA) and a polyadenylation signal (AATAAA) 36 nucleotides downstream from the termination codon. The 5′ end of the molecule was cloned following the 5′RACE method, yielding a sequence of 5440 bp (accession number in databases AJ517411).

Conceptual translation of the nucleotide sequence gave a single open reading frame encoding 1770 amino acids, with a calculated molecular mass of 201 kDa. The first 16 amino acids would correspond to a signal pep-

tide, as predicted using the SIGNALP version 2.0 computer program (Nielsen et al., 1997). There were four putative cleavage sites showing the RXXR/S consensus sequence (see Sappington and Raikhel, 1998), and three putative glycosylation sites, although only that placed at position 296–298 may be effectively glycosylated according to NetNGlyc 1.0 Prediction Server. The sequence includes a small polyserine domain (amino acids position: 372–381), and the conserved motif GLCG at position 1596–1599, followed by a number of cysteines (nine in hymenopterans) at conserved locations near C-terminal (see Chen et al., 1997; Comas et al., 2000; Lee et al., 2000a,b; Tufail et al., 2000, 2001). In addition, a DGXR motif is located 18 residues upstream of the GL/ICG motif as occurs in all insect vitellogenins studied to date (see Tufail et al., 2000 and references therein), except in that of the cockroach *L. maderae* (Tufail and Takeda, 2002).

Wheeler and Kawooya (1990) described the fragments of *A. mellifera* vitellogenin: DIQAVLKVLTEYLYLLISMI, and SIGPNWGVGNEK, which were not observed in our sequence. Only weak similarity of both was found with the fragment DFQHNWQVGNEYTYLVRSRT (position 17–36) of our sequence. A possible explanation for this apparent discrepancy is that the sequences described by Wheeler and Kawooya may correspond to another vitellogenin of *A. mellifera*. Alternatively, it might also be that the Africanized honey bee studied by us has a vitellogenin different from that of the ordinary European honey bee.

The amino acid sequence of *A. mellifera* vitellogenin was aligned with other 19 insect vitellogenins corresponding to 16 species, which revealed numerous motifs conserved in all sequences, but modest overall similarity with other sequences. The percentage of similarity of *A. mellifera* vitellogenin compared to other insect vitellogenins was between 32 and 58%, the highest values corresponding to hymenopteran species (*P. nipponica* and *A. rosae* with 58 and 54% similarity, respectively), while the lowest similarity percentages were found with cockroaches (*B. germanica* and *L. maderae*, with 32 and 33% similarity, respectively).

Finally, a phylogenetic analysis of the insect vitellogenins available (the amino acid sequences without the signal peptide) was performed following the method of Neighbor-joining. As expected, the resulting tree (Fig. 1) reflects the current phylogenetic classifications of insects.

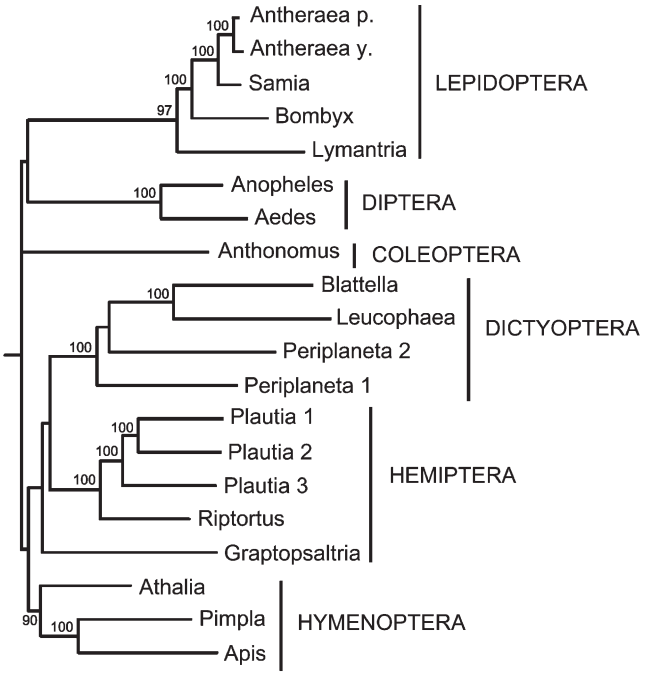

Fig. 1. Phylogenetic analysis of the amino acid sequence of insect vitellogenins (without the signal peptide) using the Neighbor-joining method. Numbers in the nodes correspond to bootstrap values (100 replicates). See the binomial nomenclature of all species and the accession number of the corresponding sequences in Section 2.

3.2. Expression of vitellogenin in queens

First, Northern blot analysis was carried out to determine the size of vitellogenin mRNA in fat body from newly emerged queens. Two fragments of *A. mellifera* vitellogenin cDNA, corresponding to sequences between nucleotides 3605–4905 and 4809–5440, were used as probes. Results obtained with these two probes were the same, and the transcript was detected as a single 5.8 kb band. The size of this mRNA is similar to those described for other insect vitellogenins (see Tufail and Takeda, 2002, and references therein).

Vitellogenin had been first detected in the hemolymph of Pbm pupal stage of queens by immunological methods; then vitellogenin levels increased during pupal development and peaked in newly emerged queen adults (Barchuk et al., 2002). To determine the onset of vitellogenin expression in queens of *A. mellifera*, total RNA was extracted from pupal abdomens at various stages of development (Fig. 2A). Vitellogenin mRNA was detected in PbmI stage, expression increased in Pbd stage, where pupal expression was maximal, and even higher in the adult fat body.

The first signs of vitellogenin gene transcription in Pbm pupal stage, coincides with still low levels of JH (which will increase thereafter) and a decline of ecdysone titers in the hemolymph (Barchuk et al., 2002). This supports the hypothesis of these authors, who suggested that low levels of JH induce vitellogenin transcription in queens of *A. mellifera*.

3.3. Expression of vitellogenin in workers

Vitellogenin had been first detected in the hemolymph of pharate adults (Pha) by immunological techniques

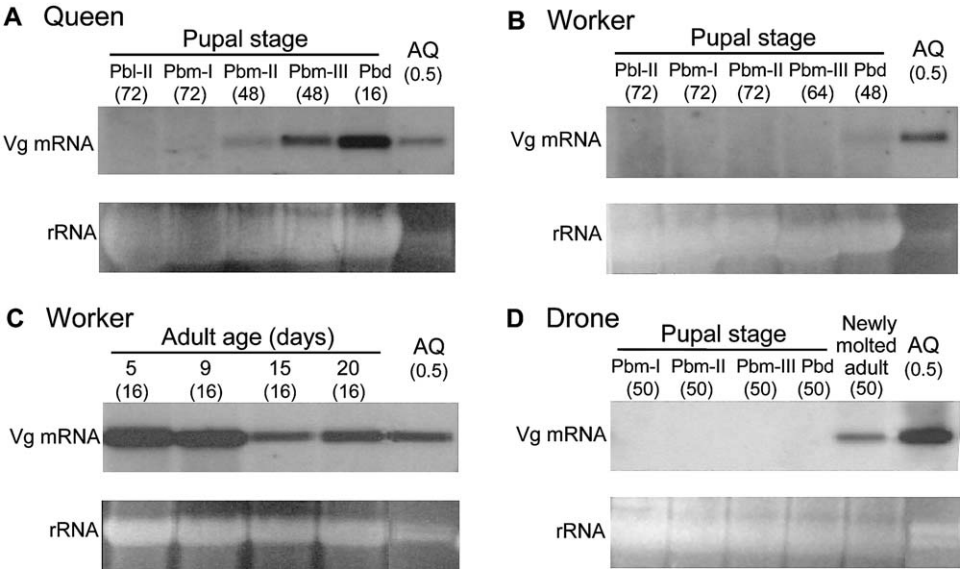

Fig. 2. Northern blot analysis of vitellogenin mRNA in *A. mellifera*. Castes and stages within the life cycle are indicated on each panel. Pupal stages are described in Table 1. In all panels the last lane corresponds to RNA from fat bodies of newly emerged adult queens (AQ), used as positive control. The number in parenthesis below the stage indicates the amount of RNA (in μg) loaded. Total RNA was extracted from entire abdomens (in pupae) or from the abdominal fat body (in adults). The portion of the gel containing rRNA was stained with ethidium bromide to control for equivalent sample loading. Each blot is representative of three replicates.

(Barchuk et al., 2002). The first traces of vitellogenin mRNA were observed late in pupal development, in Pbd stage (Fig. 2B). This is compatible with the first appearance of vitellogenin in Pha, and also with the declining ecdysone and increasing JH titers occurring at Pbd pupal stage in workers (Barchuk et al., 2002). In adult workers, vitellogenin production decreases progressively and finally ends about 20 days after the adult molt (Engels, 1987).

In order to study transcription of the vitellogenin gene in adult worker of *A. mellifera*, total RNA was extracted from the abdominal fat body of workers of 5, 10, 15 and 20 days of adult age, which had been reared in colonies in the presence of a queen. Results (Fig. 2C) show that vitellogenin mRNA levels decrease progressively with age, although they were present in all ages studied. They were still detectable, although at very low levels and only after overexposing the Northern film, in 30-day-old adult workers (not shown). In any case, vitellogenin expression in the fat body of adult workers was lower than that of adult queens (Fig. 2C), a difference that is also observed in terms of vitellogenin measured in the hemolymph with immunological methods (Barchuk et al., 2002). The synthesis of vitellogenin in workers has been explained as a mechanism of nest protection against queen loss, which would allow workers to develop their ovaries and to ensure the maintenance of the hive, at least guaranteeing the production of males (Engels et al., 1990; Oldroyd et al., 1994).

3.4. Expression of vitellogenin in drones

In *A. mellifera* drones, vitellogenin is present in the hemolymph mainly in the first days of adult age (Engels et al., 1990; Trenczek and Engels, 1986; Trenczek et al., 1989). In order to determine the onset of vitellogenin gene transcription in drones, we extracted total RNA from the abdomen of drone pupae of different stages and vitellogenin mRNA was analyzed. Vitellogenin mRNA was not detected in pupae, but it was apparent in freshly molted adult drones. Levels were, however much lower than those observed in adult queens (Fig. 2D). Vitellogenin mRNA was still detected in drones on day 4 but became practically undetectable on day 11 of adult life (not shown).

The ability of drones to synthesize vitellogenin has been attributed to the haploid mechanism of sex determination in hymenopterans, where the absence of a sex-specific locus permits a partial development of female characters in males (Engels et al., 1990; Trenczek et al., 1989). However, this explanation seems unlikely since vitellogenin is found in males of some other insects where this mechanism does not occur, like in the hemipteran *Rhodnius prolixus* (Valle et al., 1987). Within the hymenopterans, the fact that vitellogenin gene is not expressed in males of non-social species, like *A. rosae* (Kageyama et al., 1994) or *P. nipponica* (Nose et al., 1997), suggests that drone expression of vitellogenin is related in some way to social biology. Insect vitellogen-

ins have also been implicated in the transport of sugars, lipids, phosphates, vitamins and hormones (Chen et al., 1997; Sappington and Raikhel, 1998), and these may also be functions for vitellogenin in drones of *A. mellifera*.

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