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# Leucine-aminopeptidase A (LAP-A) Encoding Gene in Apoidea: from Genomic Identification to Functional Insights Based on Gene Expression

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**Abstract**

Aminopeptidases are enzymes that cleave the N-terminal region of proteins and show structural conservation in prokaryotes and eukaryotes. We aimed to identify leucine-aminopeptidase A (LAP-A) orthologs in the genome of bee species with different levels of social organization, and to explore the putative roles of this enzyme based on gene expression data. We identified a single gene for LAP-A on chromosome 15 of *Apis mellifera* L. and predicted orthologs in genomes of 11 bee species. We found evidence of LAP-A expression in more than 50 bee species. In honeybee and other bees, LAP-A transcripts were expressed in diverse tissues, including: brains, fat bodies, ovaries, testicles, integuments, and glands, on different developmental stages that spanned from embryogenesis to adult life. Our findings on the transcriptional activity of LAP-A are consistent with previously published data on enzymatic activity of LAP-A in bees throughout the development in different tissues and in both sexes. The presence of LAP-A gene in the Apoidea genomes and its ubiquitous expression support housekeeping roles of this enzyme and broad-spectrum functions in bees, independent of their life styles.

## Introduction

Aminopeptidases (α-aminoacyl-peptide hydrolase, EC 3.4.11) are ion-dependent exopeptidases that catalyze the hydrolytic cleavage of amino acid residues on the N-terminal region of (poly) peptides. There are more than twenty classes of aminopeptidases, well conserved in structure, from bacteria to humans (Sanderink et al., 1988; Matsui et al., 2006; Nandan & Nampoothiri, 2017). These classes include: leucine- (or leucyl-) aminopeptidase, membranal alanyl aminopeptidase, aminopeptidase A, aminopeptidase B, aminopeptidase N, aminopeptidase Y, cistinyl aminopeptidase, tripeptidyl aminopeptidase, prolyl aminopeptidase, glutamyl aminopeptidase and aspartyl aminopeptidase. This classification is based on the type of substrate that each aminopeptidase hydrolyzes. For instance, the aminopeptidase N hydrolyzes alanine and leucine residues, while the aminopeptidase A hydrolyzes aspartate and glutamate (Ferreira & Terra, 1984; Terra & Ferreira, 1994).

Although aminopeptidases are conserved and well described as digestive enzymes, they can perform specific physiological roles in the processing and degradation of peptides depending on the organism and tissue (McCulloch et al., 1994). In humans, for example, the perturbed activity of aminopeptidases is related with aging and pathologies (Taylor, 1993), such as hypertension (Zee et al., 2018) and cancers (Schreiber et al., 2018).

In insects, aminopeptidases are also associated with the recognition of Cry proteins (Gómez et al., 2018). Cry proteins, produced by gram-positive and entomopathogenic bacteria *Bacillus thuringiensis*, are cleaved in the midgut to form active toxins. These toxins bind to specific receptors in

the midgut, aminopeptidases being one of these receptors, producing pores that lead to cell lysis and the death of the insect (Ferré & Van Rie, 2002). Also, some strategies for controlling insect pests are based on the screening for natural inhibitors of aminopeptidases (Valencia et al., 2014). Aminopeptidases are more active than carboxypeptidases in the gut of insects (Terra & Ferreira, 1994). The aminopeptidases of insects have molecular mass between 90 e 130 KDa and present optimum activity when physiological pH ranges from 7.2 to 9.0. They have a large specificity to their substrate and, most of the time, are N aminopeptidases. The aminopeptidases in Orthoptera, Hemiptera and Coleoptera Adephaga are found in a soluble form, while aminopeptidases in Coleoptera Polyphaga, Diptera and Lepidoptera associate with the microvillar membrane of intestine cells (Terra & Ferreira, 1994).

In Hymenoptera, most studies involving aminopeptidases focused on wasps and bees. The activity of a single aminopeptidase was observed in the gut extract of the bee *Scaptotrigona bipunctata* (Lepeletier) (Schumaker et al., 1993). In the wasp *Polistes versicolor* (Olivier), five aminopeptidases were found during the development of males and females (Del Lama & Ferreira, 2003). Aminopeptidases were also identified in 14 species of bees (*Apis mellifera* L., *Melipona quadrifasciata* Lepeletier, *Melipona quinquefasciata* Lepeletier, *Melipona marginata* Lepeletier, *Melipona bicolor bicolor* Lepeletier (earlier named *Melipona nigra* Lepeletier), *Melipona scutellaris* Latreille, *Melipona rufiventris* Lepeletier, *Scaptotrigona xanthotricha* Moure, *Scaptotrigona postica* Latreille, *Scaptotrigona bipunctata* (Lepeletier), *Nannotrigona testaceicornis* (Lepeletier), *Schwarziana quadripunctata* (Lepeletier), *Plebeia droryana* (Friese) and *Friesella schrottkyi* (Friese)). In all these bee species, one aminopeptidase (named as Group 1) was always active, while the activity of other aminopeptidases varied according to species (named as Group 2, restricted to pupae with pigmented eyes) (Del Lama & Mestriner, 1984).

In honeybees (*A. mellifera*), enzymatic activities of four leucine-aminopeptidase (LAP) were reported, suggesting the existence of four different LAP-coding genes (Del Lama et al., 2001). LAP-A was invariably detected in all developmental stages (embryos, larvae, pupae and adults) and in different tissues of each gender (digestive tract, reproductive tract, cerebral ganglia, thorax, antenna and Malpighian tubules) while LAP-D, LAP-G and LAP-P showed more specific spatiotemporal activity patterns (Del Lama et al., 2001). Leucine-aminopeptidases (LAP, EC 3.4.11.1) are cytosolic proteases (Liew et al., 2013), also known as peptidase S and cytosol aminopeptidase (http://enzyme.expasy.org/EC/3.4.11.1), belonging to the (metalloexo) peptidase family M17 (NCBI: CDD cd00433 and domain architecture ID 10087321) (Matsui et al., 2006).

Comparing results from two published studies, we clearly observed that enzymatic activities of the LAP-A (Del Lama et al., 2001) and the aminopeptidase of the Group 1 (Del Lama & Mestriner, 1984) presented constitutive bands and similar gel electrophoretic patterns based on the hydrolysis of the same substrate (derivatives of beta-naphthylamine). Together, the information reinforces that observed uniform gel bands found in both studies represent the activity of the same enzyme, i.e., LAP-A. Thus, this evidence leads us to hypothesize that both mRNAs and enzymes resulting from LAP-A gene expression have housekeeping roles in cells and broad-spectrum functions in bees.

In this study, we aimed (i) to identify cytosolic LAP-A orthologs in the genome of bee species with different levels of social organization, (ii) to verify if the LAP-A gene expression profiles fit well to the previously reported LAP-A enzymatic activity and (iii) to suggest putative roles of this enzyme based on literature information and gene expression data (presence of mRNA) in different tissues and developmental stages of honeybees (*A. mellifera*) and other bees.

## Materials and Methods

### *In silico analysis*

Searches for a cytosolic leucine-aminopeptidase (LAP-A) and other aminopeptidases encoding genes of honeybees were performed in the NCBI-GenBank (http://www.ncbi.nlm.nih.gov) database, using the key words "aminopeptidase", "cytosol" and "*A. mellifera*". Resulting *A. mellifera* LAP-A nucleotide and protein sequences were recovered in FASTA format. Genomic identification (using *A. mellifera* genome data, version Amel4.5) and manual annotation of honeybee LAP-A gene structure (exon/intron) were performed using BLAST (Altschul et al., 1990) and Artemis platform (Rutherford et al., 2000), and served as guide for the design of specific LAP-A primers (Fig S1) used for gene expression analysis. *A. mellifera* LAP-A protein sequence was also used for new BLASTP rounds against both GenBank (NCBI) and BeeBase (http://hymenopteragenome.org/beebase/) databases, to search for orthologs in the genomes of other bees: Apidae (*Apis florea* Fabricius, *Apis dorsata* Fabricius, *M. quadrifasciata*, *Eufriesea mexicana* (Mocsáry), *Bombus terrestris* L., *Bombus impatiens* Cresson, *Habropoda laboriosa* Fabricius), Megachilidae (*Megachile rotundata* (Fabricius)) and Halictidae (*Lasioglossum albipes* (Fabricius), *Dufourea novaeangliae* (Robertson)). As validation criteria for orthologs, we considered the occurrence of reciprocal best BLAST hit with honeybee LAP-A protein sequence, identity threshold >75% (Mazza et al., 2009), highly significant e-value <1e-30 (Nunes et al., 2004), and also the presence of the functional domain M17 peptidase (cd00433) (analyzed by the NCBI CD-search tool, https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). In addition, digital expression based on BLASTN searches against RNA-Seq data (SRA and TSA transcriptomic collections publicly available at NCBI) from many bees was performed.

*Genomic DNA isolation*

Genomic DNA (gDNA) was isolated by the phenol-chloroform method (Sheppard & McPheron, 1991) from thorax of an individual forager worker from each bee species: *A. mellifera*, *Partamona helleri* (Friese), *Eulaema nigrita* Lepeletier, *Euglossa cordata* (Linnaeus) and *Bombus brasiliensis* Lepeletier. The DNA was then suspended in 50 µL of TE 1X buffer (Tris 10 mM, EDTA 0.1 mM, pH 8.0). Samples were quantified spectrophotometrically using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies), and diluted to 100 ng/µL (using TE 1X buffer accordingly).

*Total RNA isolation and cDNA synthesis*

The species *A. mellifera* whole bodies or dissected tissues (brain, fat body, integument, ovary, testicle) of individuals in immature stages of development (embryo, larvae and pupae) and adults, of both sexes, with females of both castes (queens and workers) were collected (according to Table S1) and pooled into individual microtubes based on sample origin. Total RNA of each pooled sample was isolated using Trizol method (Invitrogen) following the manufacturer's instructions. Samples were quantified spectrophotometrically using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies), and ~3 µg were treated with DNase I (Invitrogen) to eliminate contaminant DNA. DNase-treated samples were used as template to synthetize first strand cDNA using SuperScript II Reverse Transcriptase (Invitrogen) and oligo(dT)12-18 primers (Invitrogen) according to the manufacturer's protocol.

*PCR for gene expression analysis*

Presence or absence of the LAP-A encoding gene expression was performed using end-point Polymerase Chain Reaction (PCR). cDNA samples were amplified through conventional PCR using specific LAP-A primers, LAP-A-F (forward): 5'-AGAGGCTTTAGGAGATGCAG-3' and LAP-A-R (reverse): 5'-GCTGGAGTATCTGCCAAATG-3'. The reaction mixtures were prepared with 10 µL of Taq Pol Master Mix 2X Green (Cellco), 0.8 µL of each primer pair (10 pmols/µL), 1 µL of cDNA (dilution 1:10), in a final volume of 20 µL. Amplification reactions were assayed in the Veriti® 96-Well Thermal Cycler (Applied Biosystems), as follows: first step of 94 °C for 1 minute; 30 cycles of 94 °C for 35 seconds, 56 ºC for 35 seconds and 72 ºC for 35 seconds; and a final step at 72 ºC for 5 minutes. The expression of the RpL32 reference gene (NM_001011587.1) (Lourenço et al., 2008) was used to check the quality and the reliability of the cDNA samples; except for the use of 30 cycles and 0.5 µL of each primer pair (10 pmols/µL, RpL32-F: 5'-CGTCATATGTTGCCAACTGGT-3' and RpL32-R: 5'-TTGAGCACGTTCAACAATGG-3'), all other conditions of PCR cycling and reaction mixtures to amplify the RpL32 gene were as those used for LAP-A. Amplification products of both genes were analyzed by electrophoresis in 2% agarose gels in 1X TBE buffer (89mM Tris base, 89mM boric acid, 2 mM EDTA, pH 8.0) and stained with a Unisafe Dye solution (20,000x, Uniscience), under conditions of 55 mA and 100 volts. The bands were visualized and documented using a Kodak EDAS 290 system. Each gel band volume (the sum of all pixel intensities within the band boundaries excluding background signs) was densitometrically measured by ImageJ version 1.52a software (Abramoff et al., 2004), and LAP-A/RpL32 ratio was calculated to normalize LAP-A transcripts abundance.

*Sequencing*

For sequencing, 1 µL DNA sample (100 ng/µL) from *A. mellifera*, *E. nigrita, P. helleri*, *B. brasiliensis* and *E. cordata* was subjected to amplification, using 2.5 µL of dNTP (250 mM), 2.5 µL of buffer 10X (Invitrogen), 2.5 µL of MgCl2 (50 mM, Invitrogen), 1 µL of each LAP-A primer (10 pmols/µL, LAP-A-F or ), 0.2 µL of Taq Platinum® (5 U/µL, Invitrogen), in a final volume of 25 µL. Amplification reactions were assayed in the Veriti® 96-Well Thermal Cycler (Applied Biosystems), as follows: first step of 95 °C for 3 minutes; 35 cycles of 95 °C for 30 seconds, 56 ºC for 45 seconds and 72 ºC for 45 seconds; and a final step at 72 ºC for 10 minutes. Amplification products were analyzed by electrophoresis in 1% agarose gels in 1X TBE buffer and stained with a Gel Red solution (10000x, Biotium), under conditions of 55 mA and 100 volts. The bands were visualized and documented using a Molecular Image Gel Doc™ XR+ photodocumenter (Bio-Rad) and Image Lag 4.0 software (Bio-Rad). Concerning the DNA sequencing, a single and intense band was obtained for *A. mellifera*. However, some unspecific bands were observed for other species. The bands compatible with the expected fragment size based on the LAP-A gene fragment from *A. mellifera* (278 bp) were cut from the gel and placed in separate tubes with 200 µL of sterilized water. The tubes were heated at 70 °C for 30 minutes to partially melt the agarose and facilitate the passage of DNA to water. From these samples, a second round of PCR were performed under the same conditions (cycle number and reagents), only modifying the annealing temperature to 62 °C, to increase specific amplification. The new amplification products were purified using 2 µL of ExoProStarTM (IllustraTM) enzyme and 8 µL of gDNA amplified in each reaction. The purification reaction was incubated in the Veriti® 96-Well Thermal Cycler (Applied Biosystems) at 37 °C for one hour, at 80 °C for 15 minutes and at 20 °C for 5 minutes. Next, an aliquot of this reaction was submitted to 1% agarose gel electrophoresis under the conditions cited above. Purified PCR products that showed single and clear bands for each species were selected for sequencing in both directions. The sequencing reaction used 3 µL of sterilized water, 3 µL of buffer Save Money 2.5x, 1 µL of Big Dye Terminator version 3.1 (Thermo Fisher Scientific), 1 µL of forward or reverse LAP-A primer and 2 µL of PCR purified product per well in the sequencing 96-well plate, with a final volume of 10 µL. The reaction was conducted in 40 cycles: 96 °C for 1 minute, 50 °C for 15 seconds, 62 °C

for 15 seconds and 60 °C for 4 minutes. For precipitation, washing steps using 75% isopropanol (v/v) and 70% ethanol (v/v) were used. After centrifugations, the 96-well plate was incubated at 37 °C for 10 minutes and sent to Laboratório de Biotecnologia of FCAV/UNESP (Jaboticabal, SP, Brazil) for sequencing. The sequencing reactions were assayed in an ABI 3700 automatic sequencer (Applied Biosystems) and the electropherograms were analyzed and edited in Codoncode version 3.7.1 program (CodonCode).

## Results

*LAP-A genomics*

We identified 22 potential aminopeptidases encoding genes in the *A. mellifera* genome and 50 splicing variants, distributed in eight of the sixteen chromosomes (haploid genome) (Table S2). Our analysis resulted in the identification of a single gene model noted as being an *A. mellifera* cytosolic leucine-aminopeptidase encoding gene (LAP-A) (GeneID: 552109 and GenBank accession number: XM_016916769.1). LAP-A gene locus is located in chromosome 15, and its full-length mRNA of 1,617 base pairs (bp) contains 46 bp of 5'UTR, 86 bp of 3'UTR, and 1,485 bp of coding region (CDS) that generates a protein with 494 amino acids (GenBank accession number: XP_016772258.1). It is worthwhile mentioning that the *A. mellifera* LAP-A protein sequence has 53% identity (score: 494, query cover: 94%, e-value: 5e-67) with human cytosol aminopeptidase (NP_056991.2).

The honeybee LAP-A sequences in FASTA format were recovered from GenBank, and served as a starting point to search for orthologs in genomes of other bee species (Apoidea). We found predicted orthologs of LAP-A from solitary to social species: Apidae (*A. dorsata*, *A. florea*, *M. quadrifasciata*, *E. mexicana*, *B. terrestris*, *B. impatiens*, *H. laboriosa*), Megachilidae (*M. rotundata*), and Halictidae (*L. albipes*, *D. novaeangliae*) (Table 1). In addition, a coherent evolutionary relationship between LAP-A of bees was obtained (Fig S2) when we aligned only central protein regions (~334-338 amino acid residues) encompassing their conserved Peptidase_M17 domains (cd00433).

Using specific LAP-A primers, we amplified fragments of 278 bp and 201 bp in length using *A. mellifera* gDNA and cDNA samples as template, respectively (Fig. S1, and lanes 1 and 2 of Fig S3). We also used these primers to amplify and sequence the LAP-A gene from gDNA samples of the following corbiculated bees: *P. helleri*, *E. nigrita*, *E. cordata* and *B. brasiliensis*. PCR products of *A. mellifera* LAP-A were (re)sequenced only for validation, as its genome is already known. In general, the results of sequencing were incomplete, but satisfactory. Sequencing of putative LAP-A fragments from *P. helleri* and *B. brasiliensis* did not present sufficient quality and length for analyzes, while those sequences from *A. mellifera*, *E. nigrita* and *E. cordata* were reliable (see Fig S3) and aligned with LAP-A, as expected (Table S3).

**Table 1**. Orthologs of *Apis mellifera* LAP-A protein sequence (XP_016772258.1), identified by BLASTP tool against genomic data of Apoidea species available at NCBI or BeeBase. Highlighted in bold, corbiculated bee species from Apini, Meliponini, Euglossini and Bombini tribes.

| Species | Taxonomy (Family/Tribe) | Life style | Accession number | Identity in relation to *A. mellifera* | e-value in relation to *A. mellifera* |
|---|---|---|---|---|---|
| ***Apis mellifera*** | **Apidae/ Apini** | **advanced eusocial** | **XP_016772258.1** | **100%** | **0.0** |
| ***Apis dorsata*** | **Apidae/ Apini** | **advanced eusocial** | **XP_006609318.1** | **99%** | **0.0** |
| ***Apis florea*** | **Apidae/ Apini** | **advanced eusocial** | **XP_012345156.1** | **98%** | **0.0** |
| ***Melipona quadrifasciata*** | **Apidae/ Meliponini** | **advanced eusocial** | **KOX72754.1** | **91%** | **0.0** |
| ***Eufriesea mexicana*** | **Apidae/ Euglossini** | **facultative simple eusocial** | **XP_017752846.1** | **92%** | **0.0** |
| ***Bombus terrestris*** | **Apidae/ Bombini** | **obligate simple eusocial** | **XP_003401321.1** | **90%** | **0.0** |
| ***Bombus impatiens*** | **Apidae/ Bombini** | **obligate simple eusocial** | **XP_003484843.1** | **90%** | **0.0** |
| *Habropoda laboriosa* | Apidae/ Anthophorini | solitary | XP_017799497.1 | 87% | 0.0 |
| *Megachile rotundata* | Megachilidae/ Megachilini | solitary | XP_012141098.1 | 85% | 0.0 |
| *Lasioglossum albipes* | Halictidae/ Halictini | facultative simple eusocial | LALB20051 (BeeBase ID) | 81% | 0.0 |
| *Dufourea novaeangliae* | Halictidae/ Rophitini | solitary | XP_015439447.1 | 85% | 0.0 |

*LAP-A mRNA expression*

We explored LAP-A transcriptional expression data in different species, tissues and developmental stages in order to gain functional insights and to verify the correspondence between mRNA profiles and enzymatic activity. Toward this end, two strategies were used: digital expression using BLAST searches against all currently TSA and SRA (transcriptomic) data available for Apoidea (Table S4), and semiquantitative RT-PCR assays using honeybee cDNA samples (Fig 1 to Fig 6).

Regarding digital expression, more than 50 different bee species belonging to six distinct taxonomic Apoidea families (Apidae, Megachilidae, Halictidae, Melittidae, Colletidae and Andrenidae) presented some transcriptional evidence for LAP-A (Table S4), corroborating our hypothesis about its constitutive expression and general roles in bees. About RT-PCR data, the reference gene RpL32 provided us information about the quality and the reliability of the samples (Fig S4 to Fig S9), as well as served to normalize the abundance of LAP-A transcripts. Even though some fluctuations in LAP-A expression are sometimes observed in the different analyzed contexts (Fig S10), its presence was invariably found in all of them.

*Embryogenesis*

Our findings indicate that LAP-A is expressed throughout the embryogenesis of both honeybee sexes (Fig 1), increasing towards late embryogenesis (Fig S10). RNA-Seq data also supported the expression of LAP-A during the embryonic development of *A. mellifera*, *Apis cerana* Fabricius, *Bombus hunti* Greene and *Megalopta genalis* Meade-Waldo (Table S4).

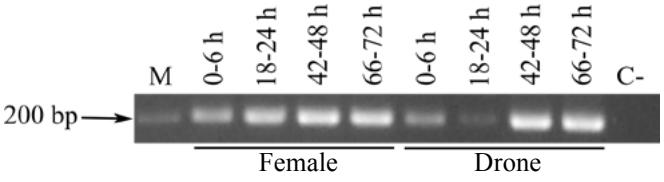


**Fig 1.** Expression of LAP-A (cDNA, 201 bp) in male (drone) and female embryos throughout the embryogenesis (time interval in hours). In M, molecular weight marker and in C-, negative control reactions with no added template (cDNA). PCR products were visualized in agarose (2%) gel electrophoresis. For information of each sample, see Table S1.

*Post-embryonic development*

We observed the expression of LAP-A during entire larval development of males and females of *A. mellifera* (Fig 2 and Fig 3), and data hint specific sex profiles (Fig S10). From TSA/SRA data, we detected expression of LAP-A in larvae of *A. mellifera*, *B. terrestris*, *B. hunti*, *M. rotundata* and *M. genalis*, and in pupal tissues of *A. mellifera*, *B. terrestris*, *B. hunti* e *M. genalis* (Table S4).

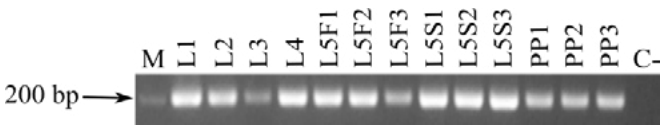


**Fig 2.** Expression of LAP-A (cDNA, 201 bp) in whole bodies of bipotent female larvae (L1 and L2, collected from worker brood cells) and worker larvae (L3 to PP3). In M, molecular weight marker and in C-, negative control reactions with no added template (cDNA). PCR products were visualized in agarose (2%) gel electrophoresis. For information of each sample, see Table S1.

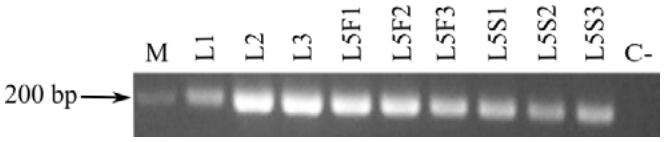


**Fig 3.** Expression of LAP-A (cDNA, 201 bp) in whole bodies of developing drone larvae (L1 to L5S3). In M, molecular weight marker and in C-, negative control reactions with no added template (cDNA). PCR products were visualized in agarose (2%) gel electrophoresis. For information of each sample, see Table S1.

*Differentiation of reproductive organs*

LAP-A transcripts were also detected in the ovaries and testicles during post-embryonic development of *A. mellifera*, when the differentiation of these reproductive tissues occurs (Fig 4). In addition, RNA-Seq data also support expression of LAP-A transcripts in ovaries of queens and workers undergoing distinct reproductive status in *A. mellifera* and *B. terrestris*, and in drone testicles of the same two species (Table S4).

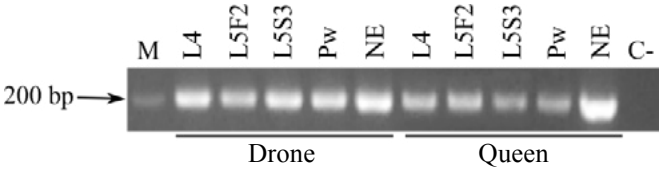


**Fig 4.** Expression of LAP-A (cDNA, 201 bp) in drone testicles or queen ovaries during different developmental stages. In M, molecular weight marker and in C-, negative control reactions with no added template (cDNA). PCR products were visualized in agarose (2%) gel electrophoresis. For information of each sample, see Table S1.

*Adults*

We verified a subtle differential expression of LAP-A between brains and fat bodies of 7 days-old adult honeybee workers (Fig 5, Fig S10). We also observed LAP-A expression in *A. mellifera* transcriptomic data from samples of abdomen, antennae, head, mushroom body, fat body, hypopharyngeal gland, second thoracic ganglia, sting gland, Nasonov gland, muscle, mandibular gland, midgut, Malpighian tubule collected in different sexes and castes (Table S4). Moreover, many bee species showed expression of LAP-A in head, abdomen, thorax and antennae, or in whole body during the adult phase (Table S4).

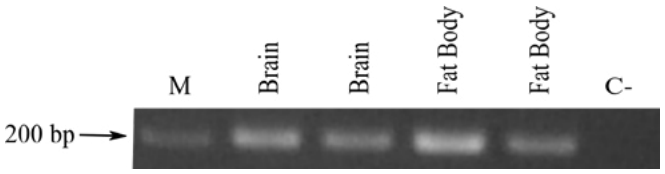


**Fig 5.** Expression of LAP-A (cDNA, 201 bp) in 7-day-old worker brains and fat bodies. In M, molecular weight marker and in C-, negative control reactions with no added template (cDNA). PCR products were visualized in agarose (2%) gel electrophoresis. For information of each sample, see Table S1.

*Integument*

LAP-A is found to be expressed in the abdominal and thoracic integument (epidermis plus cuticle) of honeybee pupae (Pw), pharate adults (Pp) and adult workers (NE and For) (Fig 6), showing a peak in Pp (Fig S10).

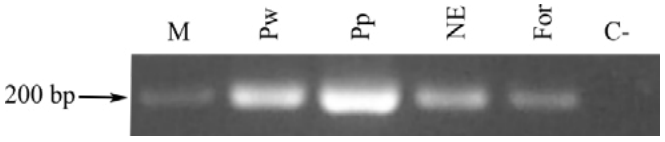


**Fig 6.** Expression of LAP-A (cDNA, 201 bp) in integument (thoracic and abdominal) of workers during different stages of development. In M, molecular weight marker and in C-, negative control reactions with no added template (cDNA). PCR products were visualized in agarose (2%) gel electrophoresis. For information of each sample, see Table S1.

## Discussion

Here we identified LAP-A orthologs in Apoidea genomes and the tree topology generated (Fig S2) fits well the proposal for bee phylogeny currently accepted (see Kapheim et al., 2015). Also, we found expression of LAP-A in all conditions tested by semiquantitative RT-PCR assays using honeybee (*A. mellifera*) cDNA samples and also found evidence of expression using BLAST searches against all currently TSA and SRA (transcriptomic) data available for Apoidea. Together, our data support the coherence between mRNA and enzyme activity profiles of LAP-A (Del Lama & Mestriner, 1984; Del Lama et al., 2001).

Below we suggested and discussed putative roles of this enzyme in different tissues and developmental stages of honeybees and other bees, based on literature information and gene expression data.

The increasing of expression of LAP-A towards late embryogenesis (Fig 1) is probably related to eggshell break for larval hatching. To date, LAP-A was the only active leucine-aminopeptidase found in eggs of *A. mellifera*, suggesting roles in yolk protein digestion (Del Lama et al., 2001). LAP-A transcripts found during early embryogenesis of honeybees (0-6h, Fig 1) may also indicate their maternal deposit and expression after genome activation, probably acting during cleavage divisions (Pires et al., 2016).

During larval stages, intense feeding requires digestive enzymes capable of hydrolyzing several protein substrates. LAP is an important enzyme present in the gut of many insects (for review, see Bozić et al., 2007). We detected transcripts of LAP-A in developing larvae of both honeybee sexes (Fig 2 and Fig 3). It is in accordance with LAP-A enzymatic activity found in larvae of 2-6 days (Del Lama et al., 2001). Moreover, metalloaminopeptidase has been associated with the larval molting process in invertebrates (Hong et al., 1993). It is tempting to speculate that this aminopeptidase may also act in the degradation of proteins that anchor the cuticle, facilitating apolysis and ecdysis (shedding of the old cuticle). Furthermore, imaginal (pupal-to-adult) molting depends on the replacement of the old cuticle by a newly synthesized version. LAP-A enzymatic activity was observed in young and pigmented pupae of honeybees (Del Lama et al., 2001). In addition, the peak of LAP-A found in the Pp integument (Fig 6) coincides with the ecdysteroid peak that induces pupal cuticle apolysis and the onset of adult cuticle synthesis in *A. mellifera* (Pinto et al., 2002; Elias-Neto et al., 2009). Aminopeptidases were found in integument of *Drosophila melanogaster* Meigen (Knowles & Fristrom, 1967), and there are reports of an increase in LAP concentration in the epidermis of arthropods during the molting process. Evidence suggests that epidermal cells of bees secrete LAP-A to the molting fluid that fills the ecdysial space (Spearman, 1973).

We detected LAP-A transcripts during the differentiation of reproductive organs of *A. mellifera* (Fig 4), as well as in females (queen and worker) ovaries and in male testicles of adult individuals of *A. mellifera* and *B. terrestris* (Table S4). LAP-A enzyme is active in reproductive tracts of adult drones (testes, mucus glands, ejaculatory bulb and seminal vesicles) and also in both virgin and inseminated queens (ovaries/oviduct and spermatheca) (Del Lama et al., 2001), corroborating our data. In the tick *Haemaphysalis longicornis* Neumann, LAP was observed in different ovary cells responsible for the nutrition of developing oocytes (Hatta et al., 2010). The same authors also silenced the expression of LAP gene and observed a negative impact in reproduction due to reduced oviposition. The data suggest a role of LAP in the metabolism of nutrients that support vitellogenesis. Also, high expression of LAP was detected in sperm proteome of *D. melanogaster* Meigen (Dorus et al., 2006). It is believed that LAP is present in the acrosome of sperm, an organelle that contains many digestive enzymes involved in the process of egg fertilization (Togo & Morisawa, 2004). Considering the high conservation of aminopeptidases, we assume that the LAP functions (linked to reproductive processes) described above may be preserved in bees.

In adults, the activity of LAP-A was found in digestive tract, thorax, antennae, hemolymph, Malpighian tubule and brain of *A. mellifera* (Del Lama et al., 2001). Here, we showed LAP-A mRNA expression in dozens of tissues from different species of bees (Fig 5, Fig S10, Table S4). Together, our data reinforce the hypothesis of pervasive expression and housekeeping roles of this aminopeptidase in cells and broad-spectrum functions in bees.

*Concluding remarks*

The constant presence of LAP-A mRNA expression presented here are consistent with LAP-A enzymatic activity previously published (Del Lama & Mestriner, 1984; Del Lama et al., 2001). Our data demonstrate that LAP-A is a ubiquitously expressed gene at transcriptional level, with potential functions in many Apoidea species. It is postulated that, in addition to the digestive role, aminopeptidases have metabolic roles, such as the regulation of circulating concentrations of amino acids and peptides in the body, maintenance of osmotic stability and delivery of amino acids for protein anabolism (see Del Lama & Ferreira, 2003). Also, LAP-A plays roles in the regulation of synthesis and degradation of intracellular proteins (protein turnover) (Miller, 1987; Yen et al., 1980; Bartling et al., 1992). Our findings support the multifunctional aspects of this enzyme acting in distinct biological processes of bees, independent of their life styles.

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**Supplementary Material**

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**Authors' Contributions**

The study was conceived and coordinated by MADL and FMFN. LB and ICG conducted the lab experiments. LB, MADL and FMFN analyzed the data and wrote the manuscript. All authors contributed to discussions of the data and edited the manuscript. All authors approved the final manuscript version.

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