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Expression profile of a Laccase2 encoding gene during the metamorphic molt in *Apis mellifera* (Hymenoptera, Apidae)

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ABSTRACT. Expression profile of a Laccase2 encoding gene during the metamorphic molt in *Apis mellifera* (Hymenoptera, Apidae). Metamorphosis in holometabolous insects occurs through two subsequent molting cycles: pupation (metamorphic molt) and adult differentiation (imaginal molt). The imaginal molt in *Apis mellifera* L. was recently investigated in both histological and physiological-molecular approaches. Although the metamorphic molt in this model bee is extremely important to development, it is not well-known yet. In the current study we used this stage as an ontogenetic scenario to investigate the transcriptional profile of the gene *Amlac2,* which encodes a laccase with an essential role in cuticle differentiation. *Amlac2* expression in epidermis was contrasted with the hemolymph titer of ecdysteroid hormones and with the most evident morphological events occurring during cuticle renewal. RT-PCR semiquantitative analyses using integument samples revealed increased levels of *Amlac2* transcripts right after apolysis and during the subsequent pharate period, and declining levels near pupal ecdysis. Compared with the expression of a cuticle protein gene, *AmelCPR14*, these results highlighted the importance of the ecdysteroid-induced apolysis as an ontogenetic marker of gene reactivation in epidermis for cuticle renewal. The obtained results strengthen the comprehension of metamorphosis in *Apis mellifera*. In addition, we reviewed the literature about the development of *A. mellifera*, and emphasize the importance of revising the terminology used to describe honey bee molting cycles.

KEYWORDS. apolysis; cuticle; ecdysteroids; honey bee; pupation.

Metamorphosis consists in a drastic reorganization of development in which an immature larva becomes a reproductively active adult (Gilbert *et al.* 1996). The metamorphic process in insects occurs in the context of growth cycles characterized by molts, *i.e.*, the periodic substitution of the old cuticle by a new one, newly-synthetized. After a series of larval molts, which number is variable among species, the larval-imaginal transition in holometabolous insects occurs through two molting cycles: one leading to pupation (metamorphic molt) and the other ultimately resulting in differentiation of the adult (imaginal molt).

The integument of insects consists of an external cuticle, or exoskeleton, that overlies the epidermis (Hepburn 1985). Each molting cycle begins with apolysis, the freeing (detachment) of the epidermal cells from the old exoskeleton (Jenkin & Hinton 1966) and finishes with ecdysis, the eventual shedding of non-profitable portion of cuticle. The morphogenetic period between apolysis and ecdysis is designated pharate (cloaked), which is the phase of an instar enclosed within the cuticle of the previous instar. Exoskeleton differentiation occurs essentially during the pharate period (Hinton 1946).

The sequence of molting events, or molting dynamics, is coordinated by the titer of ecdysteroid hormones (Riddiford 1985; Nijhout 1994) and includes a complex process of cuticle hardening, or sclerotization, in which laccase enzymes have an essential role. Laccases [(EC 1.10.3.2), *p*-diphenol: O_2 oxidoreductases] catalyze the oxidative conjugation of catechols with cuticular proteins (Kramer *et al.* 2001; Suderman *et al.* 2006), necessary for cuticle sclerotization. Laccases are supposed to be universally distributed in most various life domains (Claus 2004). They have aroused interest by their industrial applications (Durán *et al.* 2002) and, in case of their studies in insects, by their biotechnological potential in agricultural pests and disease vectors control (Arakane *et al.* 2005).

An increasing attention has been paid to insect laccases. Its enzymatic activity has been characterized in the integument of Diptera (Barrett & Andersen 1981; Barrett 1987a,b; Binnington & Barrett 1988; Sugumaran *et al.* 1992) and Lepidoptera (Dittmer *et al.* 2009). In addition, the expression of genes encoding laccases has also been described in Diptera (Gorman *et al.* 2008), Lepidoptera (Dittmer *et al.* 2004; Yatsu & Asano 2009), Coleoptera (Arakane *et al.* 2005; Niu *et al.* 2008), and more recently, in Hymenoptera (Elias-Neto *et al.* 2010) and Hemiptera (Futahashi *et al.* 2010, 2011).

If focused on the context of the periodic molts, studies on enzymes and proteins involved in cuticle formation should contribute to a better understanding of metamorphosis at the molecular level. With this goal, we characterized the activity and biochemical properties of a phenoloxidase involved in cuticle pigmentation (Zufelato *et al.* 2004), and the gene encoding this enzyme (Lourenço *et al.* 2005). In addition, we described the structure and expression of the genes encoding Laccase2 (Elias-Neto *et al.* 2010) and a structural cuticle protein, AmelCPR14 (Soares *et al.* 2007). In these studies, mostly centered on the pupal-to-adult (imaginal) molt, gene expression was approached in the context of the ecdysteroid-regulated molting events.

The purpose of the current study was to describe the temporal expression pattern of the *Amlac2* gene during the larval-to-pupal (metamorphic) molt of *Apis mellifera* Linnaeus, 1758 at the light of the ecdysteroid titer modulation during this stage (Rachinsky *et al.* 1990). In an attempt to highlight the general expression pattern of cuticle genes during molting cycles, we contrasted the expression of *Amlac2* and *AmelCPR14* (Soares *et al.* 2007) during the metamorphic and imaginal molts. This analysis was carried out in the context of the most evident ecdysteroid-regulated molting events, *i.e.*, apolysis, cuticle renewal in the pharate period, and ecdysis.

Finally, we reviewed the literature about the development of *A. mellifera*, and based on this review, we proposed a reconsideration of the use of the apolysis process as an ontogenetic mark and the replacement of the term "pre-pupa" by "pharate pupa".

MATERIAL AND METHODS

Honey bees. 5th *instar* larvae and pharate pupae of Africanized *A. mellifera* workers were obtained from hives maintained at the Experimental Apiary of the *Universidade de São Paulo*, Ribeirão Preto, SP, Brazil. Developing bees were identified as feeding larvae (L5F), spinning larvae (L5S), when they stop feeding in preparation for the metamorphic molt, and pharate pupae (PP). Subphases within L5F, L5S and PP were identified according to criteria established by Michelette & Soares (1993).

Developmental profile of *Amlac2*. Pools of larvae and pharate pupae were separately homogenized in TRIzol reagent (Invitrogen) for total RNA extraction, according to the protocol recommended by the manufacturer. The extracted RNA was incubated in the presence of RNase-freeDNAse I (Promega) for 30 min at 37°C to eliminate contaminating DNA. The first strand cDNA was synthetized from a standard amount of total RNA (6 µg), using the synthesis system of SuperScript II (Invitrogen). For cDNA amplification by PCR, we used Master Mix (Eppendorf) and specific primers to *Amlac2* gene (forward: 5' GGT ACG CAC TTC TGG CAC G 3' and reverse: 5' CGT CAT GAA ACC GGT GTT G 3'), designed from the predicted sequence (GB11321) in the assembled honeybee genome (The Honeybee Genome Sequencing Consortium 2006). The 273 bp cDNA fragment, flanked by the *Amlac2* specific primers was amplified using the following conditions: 2 min at 95ºC, 30 cycles (30 s at 94ºC, 45 s at 58ºC, and 50 s at 72ºC), and a final extension of 10 min at 72ºC. This cDNA fragment was sequenced (Elias-Neto *et al.* 2010) to confirm gene identity. *Amlac2* sequence was deposited in GenBank under the accession number FJ470292.

The cDNA loading control was carried out using specific primers for the gene encoding a cytoplasmic actin (*Amact*) (GenBank accession number AB023025), which is constitutively expressed during development (Lourenço *et al.* 2008) (forward: 5' TGC CAA CAC TGT CCT TTC TG 3' and reverse: 5' AGA ATT GAC CCA CCA ATC CA 3'). The thermal cycling program used for amplification of *Amact* cDNA was the same as described above, except for primer annealing temperature (62ºC) and number of cycles (27). The number of cycles of PCRs was tested for both cDNA sequences (*Amlac2* and *Amact*) and defined in order to avoid saturation. The amplification products were analyzed by electrophoresis in 1% agarose gels containing ethidium bromide. EDAS 290 (KODAK) was used for image analysis.

RESULTS AND DISCUSSION

Developmental profile of *Amlac2* gene expression. Bee ontogenesis involves the general pattern of holometabolous development, though associated to extremely complex social interactions between brood and adults. The larva undergoes four successive molts, marked by intervals of growth due to abundant feeding. In the fifth larval stage, each brood cell is sealed by a wax operculum produced by worker bees. The larva then stops eating, empties its gut and spins a cocoon, in preparation for the metamorphosis (Michelette & Soares 1993). The metamorphic molt starts with apolysis, proceeds through the pharate period and ends at pupal ecdysis.

We assessed by means of semiquantitative RT-PCR the pattern of *Amlac2* expression during the metamorphic molt in *A. mellifera*, which was contrasted to the ecdysteroid titer variation in hemolymph as determined by Rachinsky *et al.* (1990) (Fig. 1). The results clearly evidenced that at a certain ecdysteroid titer threshold the level of *Amlac2* transcripts notably increases, coinciding with apolysis and onset of cuticle renewal. *Amlac2* expression remained high during the pharate period when the pupal cuticle is being deposited, but rapidly declined just before the pupal ecdysis.

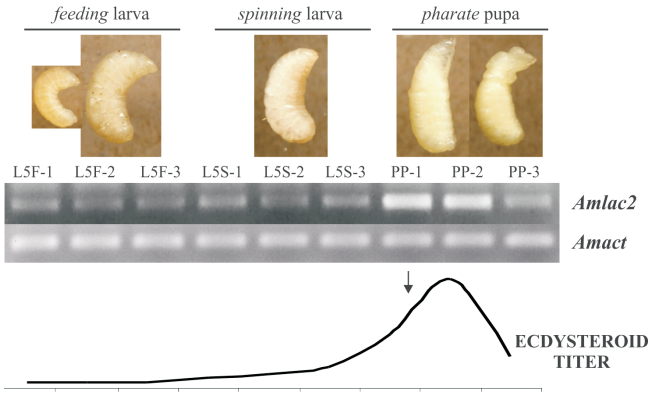

Fig. 1. Differential transcription of *Amlac2* in 5th *instar* larvae and pharate pupae in *Apis mellifera.* L5F-1, L5F-2, L5F-3: successive feeding phases. L5S-1, L5S-2, L5S-3: successive spinning phases. PP-1, PP-2, PP-3: successive pharate pupae phases. Semiquantitative RT-PCR visualized in agarose gel 1% containing ethidium bromide. Apolysis is indicated by an arrow. The ecdysteroid curve was redrawn from Rachinsky *et al.* (1990).

A similar *Amlac2* transcription pattern was observed at the subsequent imaginal molt cycle (Elias-Neto *et al.* 2010). Therefore, independently of the type of the molt, whether larval-to-pupal or pupal-to-adult, the expression of *Amlac2* is induced

concomitantly with the increase in ecdysteroid titer that triggers apolysis and cuticle renewal. This is consistent with the function of Laccase2 in cuticle sclerotization, a process occurring during the pharate period of each molt episode.

Other insect models showed a similar transcription pattern of the gene encoding Laccase2. In the lepidopteran *Manduca sexta*, the gene *Mslac2* showed the higher expression in pharate pupae in comparison to the feeding/wandering larvae and 0-day pupae (Dittmer *et al.* 2004). Similarly, in the coleopteran *Tribolium castaneum*, the highest levels of *TcLac2* transcripts were detected in pharate pupae and pharate adults (Arakane *et al.* 2005). In these studies, however, the expression profiles were not correlated with the respective ecdysteroid titers.

Reviewing metamorphosis terminology of honey bee: a morphogenetic approach. In addition to *Amlac2*, other genes with roles in cuticle formation and differentiation are induced during apolysis. As an example, the gene encoding a structural cuticle protein, *AmelCPR14* (Soares *et al.* 2007), showed induced expression associated to the increased ecdysteroid titer that triggers larval-to-pupal and pupal-to-adult apolyses (Fig. 2).

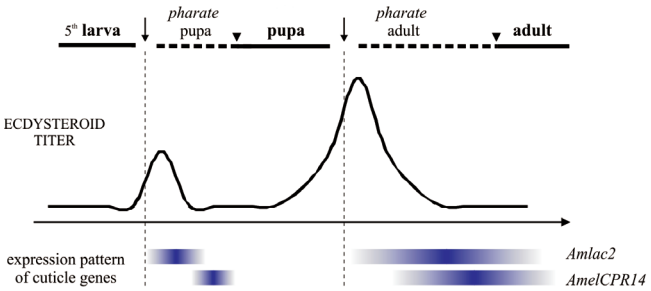

Fig. 2. Ontogenetic scenario for metamorphosis in the honey bee. The ecdysteroid hormone induces apolysis and coordinates gene expression (in blue). Simplified transcriptional patterns of the cuticle genes encoding the enzyme Laccase2 (Elias-Neto *et al.* 2010 and the present work) and the structural protein AmelCPR14 (Soares *et al.* 2007). Ecdysteroid titers were redrawn from Rachinsky *et al.* (1990) and Pinto *et al.* (2002). Apolyses are indicated by arrows and ecdyses by arrowheads.

By comparing the course of honey bee development with the modulation of expression of genes involved in cuticle renewal, it becomes clear that apolysis is a hallmark of the intense morphological, physiological and genetic changes that allow the passage to the next stage. Remarkably, the importance of apolysis in ontogenesis has been systematically neglected. The same does not happen with ecdysis, which is conveniently referred in literature as the end point of each molting cycle.

Table I compiles the honey bee literature regarding to the use of apolysis and ecdysis events as ontogenetic marks. As exceptions, Snodgrass (1956), Thompson (1978) and more recently Elias-Neto *et al.* (2009) recognized the importance of both processes. Another point also related to studies on development is the use, by the respective authors, of the term 'prepupa' instead of the more informative 'pharate pupa'. The term prepupa has prevailed over the pharate pupa, with the exception of Thompson (1978) and Elias-Neto *et al.* (2009).

The criterion adopted in this work consists in using both apolyses and ecdyses as marks of development, the first delimitating the ontogenetic status and the last the life stage. Besides, the term prepupa is inappropriate and must be replaced by pharate pupa. This developmental phase corresponds to an ontogenetic sub-division of pupa and not to the last *instar* larva (for a detailed discussion, see Elias-Neto *et al.* 2009).

Therefore, we consider this morphogenetic approach in agreement with the reality of the insect ontogenesis. We hope that entomologists and developmental biologists that use bees or other model-insects take into account the importance of considering apolysis in their studies.

The current data add new elements related to the metamorphic molting cycle and contribute for a better comprehension of metamorphosis in *Apis mellifera*. Moreover, the results shown here gather morphologic, physiologic and genetic evidences that support the adoption of the presented criterion.

Table I. Recognition of apolysis and/or ecdysis processes as ontogenetic marks and terminology (prepupa or pharate pupa) in studies on *Apis mellifera* development. Different works and reviews under distinct approaches were considered.

Reference	Apolysis	Ecdysis	Pre-pupa	Pharate pupa	Approach
Bertholf (1925)	–	+	+	–	General development
Oertel (1930)	–	+	+	–	Histology
Myser (1954)	–	+	+	–	Histology and Morphology
Snodgrass (1956)	+	+	+	–	Morphology
Jay (1963)	–	+	+	–	Behaviour and Growth
Thompson (1978)	+	+	–	+	Histology
Rembold *et al.* (1980)	–	+	+	–	General development
Michelette & Soares (1993)	–	+	+	–	General development
Nunes-Silva *et al.* (2006)	–	+	+	–	Growth
Elias-Neto *et al.* (2009)	+	+	–	+	Histology and Morphology

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