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Unraveling the molecular determinants of caste development in the honeybee *Apis mellifera*

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Nurse honeybees feed copious amounts of royal jelly to queen larvae until they enter metamorphosis. When feeding workers, however, they switch the diet for late instar larvae from pure royal jelly to a mixture of glandular secretions with honey and pollen. Moreover, prospective queen larvae receive 10 times more food than worker larvae. As a consequence of this differential feeding the two types of larvae follow two very different developmental trajectories (Haydak 1943). This process of caste differentiation involves two kinds of alterations in the original developmental pattern: one type, which we can call incremental alterations, affects the general growth of the body or specific organs. The other type can be considered as character state alterations that result in the presence or absence of entire specific structures, such as the pollen-collecting apparatus on the hind legs, wax glands, etc. Studies aiming at disentangling the molecular determinants of this developmental plasticity in *A. mellifera* come from the early by Severson et al (1989), and then Evans and Wheeler (1999, 2000), and Corona et al (1999). In 2007, our group and that of Gro Amdam demonstrated the role of *Tor* gene in body growth and the development of some organs in this species (Patel et al 2007; Barchuk et al 2007). However, by then, it was clear that most of morphological differences between castes could represent cases of kinds of morphogenetic fields operating during larval development in response to differential feeding (an idea already present in Hepperle and Hartfelder, 2001, in relation to ovary development), all acting downstream of a kind of master regulator (e.g., *Tor*, JH, methylation, royalactin; Kucharski et al 2008; Kamakura 2011; Leimar et al 2012). Following this conception, our group focused in tackling the morphological, cellular and molecular aspects of the differential development of two of those morphogenetic fields, the hind legs structures for pollen collecting in workers (Bomtorin et al 2012), and the brain (Moda et al 2013).

Sets of "caste-specific" genes may underlie differential cuticular morphogenesis and allow for pollen-collecting behavior in workers

Using Scanning Electron Microscopy, we found that all bristles are formed and correctly positioned in worker and queen hind legs in brown-eyed pupae (Pb) just after apolysis. Interestingly, we found that the cuticle of worker hind legs is formed by polygonal scales, which contrasts with the smooth appearance of the same region in queens. In addition, bristles of the tibia in worker hind legs have a characteristic socket, usually observed in mechanoreceptors (external proprioceptors). These bristles differ strikingly with the bristles found on queen legs, which do not contain this socket aspect. A mechanoreceptor (a type of sensillum) consists of cuticular components, a sensory neuron and a sheath of cells, and it is used for the mechanical perception of external stimuli. Any mechanical force exerted on this type of sensory bristle activates nerve endings. Information on the pollen load on the corbicula would then be conveyed by these mechanoreceptors informing a pollen forager when it is time to return to the hive. The rough surface of the worker hind leg tibia (cuticle with polygonal scales), together with the pollen basket, pollen comb and pollen press, represent biological stratagems allowing for efficient pollen-collecting behavior, typical of members of this caste (from Bomtorin et al 2012).

In order to identify genes responsible for the abovementioned morphological differences between castes, we performed oligonucleotide microarray hybridization analyses comparing RNA samples from hind leg imaginal discs of queen and worker pre-pupae, which is the stage when the JH level in queens are much higher than that in workers. We got a list of 200 differentially expressed genes. The observed cuticular diphenism might be controlled by the differential expression of cuticular protein genes because queens and

workers up-regulate different sets of these genes. Workers up-regulate two RR-2 genes (out of 3 members of the CPR family, characterized by the R&R consensus motif), whereas queens up-regulate only one RR-1 and another CPR gene that could not be further classified through its *Drosophila* ortholog. Thus, the over-expression of RR-2 genes in workers and the RR-1 gene in queens might be responsible for the rough cuticle of the former and the smooth cuticle of the latter (from Bomtorin et al 2012).

This differential expression of cuticular protein genes may be governed by JH acting through the transcription factor Ftz-f1, which, we show, is more expressed in queens than in workers. Ftz-f1, an orphan nuclear receptor, is known to activate cuticular protein genes in *Drosophila*. In *A. mellifera*, JH induces the expression of *ftz-f1* in queen-destined larvae, thus possibly driving the expression of cuticular protein genes. In fact, *ftz-f1* controls the expression of honeybee cuticular protein genes, up-regulating the expression of the GB15046 gene and down-regulating GB15203. During prepupal development, *ftz-f1* is more expressed in queens legs, during a time window when JH titers are up to three times higher in queens than in workers. We could furthermore show that *ftz-f1* and the gene GB15046 are more expressed in queens and that GB15023 is highly expressed in workers at this same time of development (from Bomtorin et al 2012).

Ultrabithorax expression pattern during hind leg development coincides with bristle localization in adults

Because Dedej et al (1998) and Patel et al (2007) demonstrated that hind leg determination in honeybees occurs between the 4th and 5th larval stages and our results of morphological analyses showed that hind leg structures are completely formed in Pb, using Real Time RT-PCR, we determined the developmental transcription profiles of eight genes associated with leg morphogenesis in honeybee castes from L4 to Pw (*atx-2*, *crc*, *dac*, *gug, RfaBp, abd-A*, *dll* and *Ubx*). Unlike the other genes evaluated*, Ubx* was clearly expressed differently between castes, with higher expression in workers during pre-pupa and white-eyed pupal stages than in queens. We also showed that *Ubx* transcripts and its protein product are differentially expressed in queens and workers of *A. mellifera* during the development of caste-specific hind leg morphologies. Ubx belongs to a family of transcriptional regulators that trigger differential developmental programs along the antero-posterior axis of bilaterian animals. Besides conservation in sequence and expression domains, changes in Hox gene expression is known to give rise to new structures or even new body patterns during animal evolution, directly linking Hox gene activity with morphological diversity. A detailed analysis of specific segments of worker hind leg morphology revealed that the pollen basket is formed in a region of the tibia that is free of bristles or trichomes, and it is there where we detected high levels of Ubx in prepupal and early pupal phases. The basitarsus of adult workers shows a linear arrangement of bristles, the pollen comb, and is exactly in this region were Ubx expression was absent in certain cells with large nuclei during early pupal stages of workers. Furthermore, the hind legs of queens are covered with bristles and Ubx expression was absent in the respective tibia segment. The distribution of bristles and trichomes and their presence/absence on the hind leg are also controlled by Ubx expression in *Drosophila melanogaster* and in related species (*D. simulans* and *D. virilis*), where an interesting polymorphism was observed on the femur of the hind leg, "the naked valley". This region is characterized by high levels of Ubx expression, which is not observed in other species of the group where this region of the leg is covered by trichomes, leading to infer that development of the “naked valley” depends on Ubx expression. This region may thus be considered as ontogenetically equivalent to the pollen basket on the tibia of *A. mellifera* workers. Taken together, these data indicate that the differential expression of Ubx controls alternative appendage development as well as the acquisition of caste-specific traits in the honeybee *A. mellifera* (modified from Bomtorin et al 2012).

Heterochronic larval brain development: Morphological and molecular underpinnings

Using phalloidin/DAPI staining, cell proliferation assay by EdU, *FISH* - fluorescent *in situ* hybridization, immunocytochemistry, oligonucleotide microarray hybridization, and quantitative assays via Real-Time PCR, we have tackled the morphological and molecular characteristics of development of a second “field”,

the brain, which also appears to be under the influence of the differential feeding offered to honeybee during larval development.

Our results show that pedunculi, calyces, and antennal lobes are more developed in queen than in worker larvae. Thus, because we also demonstrated the occurrence of higher rates of cell proliferation in queens, both processes, proliferation and fasciculation, appear to be responsible for the observed differential morphogenesis of female brains between honeybee castes. A larger area corresponding to neuroblasts in the brains of queens than in workers' was also reported by Roat and Landim (2008) based on the examination of histological sections from the last stage of larval development. Similarly, Groh and Röessler (2008), reported heterochronic shifts in the development of the olfactory centers during pupal development for queens and workers. However, our data not only show that the brains of queen larvae are more developed, but also that brains of queens develop faster than those of workers, thus representing a heterochronic reflex associated with differential feeding leading to differential nervous system development in female honeybees (from Moda et al 2013).

Genome-wide expression analyses and normalized transcript expression experiments monitoring specific genes revealed 21 genes with caste-specific transcription patterns (e.g., *APC-4, GlcAT-P, fax, kr-h1* and *shot*), which encode proteins that are potentially involved in the development of brain tissues through controlling the cell proliferation rate (APC4, kr-h1) and fasciculation (GlcAT-P, fax, and shot). *PP2C*, which was one of the genes up-regulated in the worker brains (detected via microarray hybridization), belongs to a family of phosphatase genes that are involved in the regulation of stress-activated protein kinase cascades, which relay signals in response to external stimuli. In honeybees, the protein encoded by this gene might intracellularly transduce the shift in the feeding regime observed in worker larvae during the L3 stage, thus controlling the expression of downstream genes, resulting in a restriction of worker brain development during the larval phase. The other gene with a *Drosophila* ortholog that was up-regulated in the worker brain is the predicted *Aristaless-Related Homeobox* (*Al-related*) gene. In vertebrates, some members of the Aristaless family (group II) are involved in optic system development and in the proliferation and differentiation of GABAergic neurons. Because *A. mellifera* workers exhibit more facets in their compound eyes and GABAergic neurons play essential roles in olfactory memory and sensory integration, an up-regulation of Al-related proteins would allow the development of nervous structures that are fundamental for key skills in the adult bee, such as navigation and communication (from Moda et al 2013).

Among the differentially expressed genes that were up-regulated in the brains of queens, *GlcAT-P* (homolog of the mammalian glucuronyltransferase *b3gat1*) and *APC-4* deserve special attention. GlcAT-P glucuronyltransferase activity is required for proteoglycan and glycoprotein biosynthesis, which is important for the development and function of the central and peripheral nervous system. In *Drosophila*, GlcAT-P is responsible for the growth of peripheral nerves during larval development. Our microarray data showed that *GlcAT-P* was transcribed at a 5.55-fold higher level in the brains of queens than in workers during the L4 stage. This result was confirmed by RT-qPCR, which also showed higher levels of gene transcription in the brains of queens throughout the larval period. The increased GlcAT-P activity observed throughout larval development could explain the greater and more rapid fasciculation observed in the brains of queens. The other gene, *APC4*, which encodes a member of the protein complex that regulates cell cycling and dendrite-axon morphogenesis, is expressed at a 3.36–higher level in the brains of queens. Interestingly, RT-qPCR confirmed this finding and showed that this gene is transcribed at a higher level in the brains of queens during the last larval instars (L4 and L5S1, when we could also detect more cell proliferation in queen brains. The protein products of these two genes might be involved in the mechanisms leading to the differential brain development observed between honeybee castes through controlling the cell proliferation rate (APC4) and fasciculation (GlcAT-P) (from Moda et al 2013).

Fax, which was one of the genes that showed an increasing transcription profile and was differentially expressed between castes, was initially characterized based on mutations that enhanced *Abelson tyrosine kinase* (*abl*) mutant phenotypes. Subsequent studies showed that Fax interacts with different proteins

involved in axon pathfinding. The genomic sequence of *Amfax* encodes a putative protein of ~45 kD, similar to two of the largest and one of the first Fax protein variants found in *D. melanogaster*. However, the deduced amino acid sequence of *AmFax* does not show stretches of hydrophobic amino acids, thus indicating that it is unlikely to play a role in cell-cell interactions, which are processes in which the *D. melanogaster* ortholog was suggested to participate. Like *kr-h1*, *fax* is up-regulated in the brains of queens during the 5th larval instar, suggesting that it participates in the differential brain morphogenesis observed between castes, likely enhancing fasciculation and interneuronal connections (from Moda et al 2013).

The *Kr-h1* gene encodes a member of the zinc finger transcription factor family implicated in neural morphogenesis and the regulation of gene expression in response to ecdysteroids. The transcription profile of this gene in developing brains shows low levels in both castes during L3-L4 and high levels only in queens during the 5th larval instar, particularly in L5F2. In spite of showing a negative correlation with neuronal morphogenesis in developing *D. melanogaster* mushroom bodies, the observed differential transcription of this gene suggests that Kr-h1 plays a caste-specific role in brain morphogenesis during most of larval development in honeybees. Because this developmental phase is characterized by premetamorphic ecdysteroids peaks and *kr-h1* expression has been suggested to be regulated by the ecdysone signaling, the up-regulation of *kr-h1* in the brains of queens may be a response to the higher titers of ecdysteroids in this caste as a consequence of differential feeding, thus contributing to shaping the development of a larger brain queen larvae (from Moda et al 2013).

The other gene that was differentially expressed between castes, *Shot,* encodes cytoskeleton-associated proteins with binding sites for F-actin and microtubules that is required for sensory and motor axon extension in *D. melanogaster*. The *shot* gene was initially identified based on a mutation in which embryonic motoneurons fail to reach their targets and the correct axonal pathfinding of mushroom body neurons does not occur, but it has also been suggested to be involved in cell proliferation. These studies showed that clusters of neuroblasts homozygous for a mutant form of *shot* exhibit significantly reduced cell numbers. Because we observed higher rates of cell proliferation and fasciculation in the brains of queens, the higher expression of *Amshot* in fourth instar queen larvae, as indicated by RT-qPCR, in *situ hybridization* and immunostaining, suggests that this gene is a pivotal player in the gene expression cascade induced by differential feeding in honeybees, which may underlie the differential brain morphogenesis that occurs in castes of *A. mellifera* (from Moda et al 2013).

Future challenges to complete our understanding of the molecular underpinnings of caste development in honeybees in response to differential feeding include the integration of diverse systemic inputs (incremental alterations) with those relating to the various morphogenetic fields, which promote character states alterations. For details on the differential hind leg and brain morphogenesis between castes, see the following papers, whose authors are greatly acknowledged: Barchuk et al (2007), Bomtorin et al (2012), and Moda et al (2013).

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