



Research

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Ubx promotes corbicular development in *Apis mellifera*

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The key morphological feature that distinguishes corbiculate bees from other members of the Apidae family is the presence of the corbicula (pollen basket) on the tibial segment of hind legs. Here, we show that in the honeybee (*Apis mellifera*), the depletion of the gene *Ultrabithorax* (*Ubx*) by RNAi transforms the corbicula from a smooth, bristle-free concave structure to one covered with bristles. This is accompanied by a reduction of the pollen press, which is located on the basitarsus and used for packing the pollen pellet as well as a loss of the orderly arrangement of the rows of bristles that form the pollen comb. All these changes make the overall identity of workers' T3 legs assume that of the queen. Furthermore, in a corbiculate bee of a different genus, *Bombus impatiens*, *Ubx* expression is also localized in T3 tibia and basitarsus. These observations suggest that the evolution of the pollen gathering apparatus in corbiculate bees may have a shared origin and could be traced to the acquisition of novel functions by *Ubx*, which in *Apis* were instrumental for subsequent castes and behavioural differentiation.

1. Introduction

Social insects are widely recognized for their complex behaviours and division of labour within the collective. The best-studied social species is the western honeybee, *Apis mellifera*, which has three castes: the queen, drones and workers, as is the case for all other *Apis* species. Workers are characterized by having a distinct feature, the corbicula (pollen basket) that they use for packing pollen and transporting it to the beehive. The corbicula is localized on the flattened and enlarged tibia of hind legs and is found in other eusocial bees, such as bumblebees and stingless bees. By contrast, this structure is much less elaborate or completely lacking in solitary and less socially complex bee species [1]. Therefore, the evolution of the pollen basket has been recognized as a major morphological innovation in social insects [2] and is tied to the development of more complex social behaviours within the Apidae.

Morphological diversification of insect hind legs has been associated with changes in the expression of the hox gene *Ultrabithorax*, *Ubx* [3–8]. Functional studies subsequently confirmed that *Ubx* directly regulates the size of individual leg segments in a number of insects [7,9,10]. These data, coupled with recent findings showing differential expression of *Ubx* between workers and queens [8], led us to test the hypothesis that this gene may play a functional role in the development of the pollen gathering apparatus in honeybees. In addition, we examined *Ubx* expression in the bumblebee, *Bombus impatiens*, to infer the generality of corbicular development in Apidae.

2. Material and methods

(a) RNA interference

Double-stranded RNA was prepared following a previously established protocol as described in Mahfooz *et al.* [9]. In short, two non-overlapping *Ubx* fragments directed

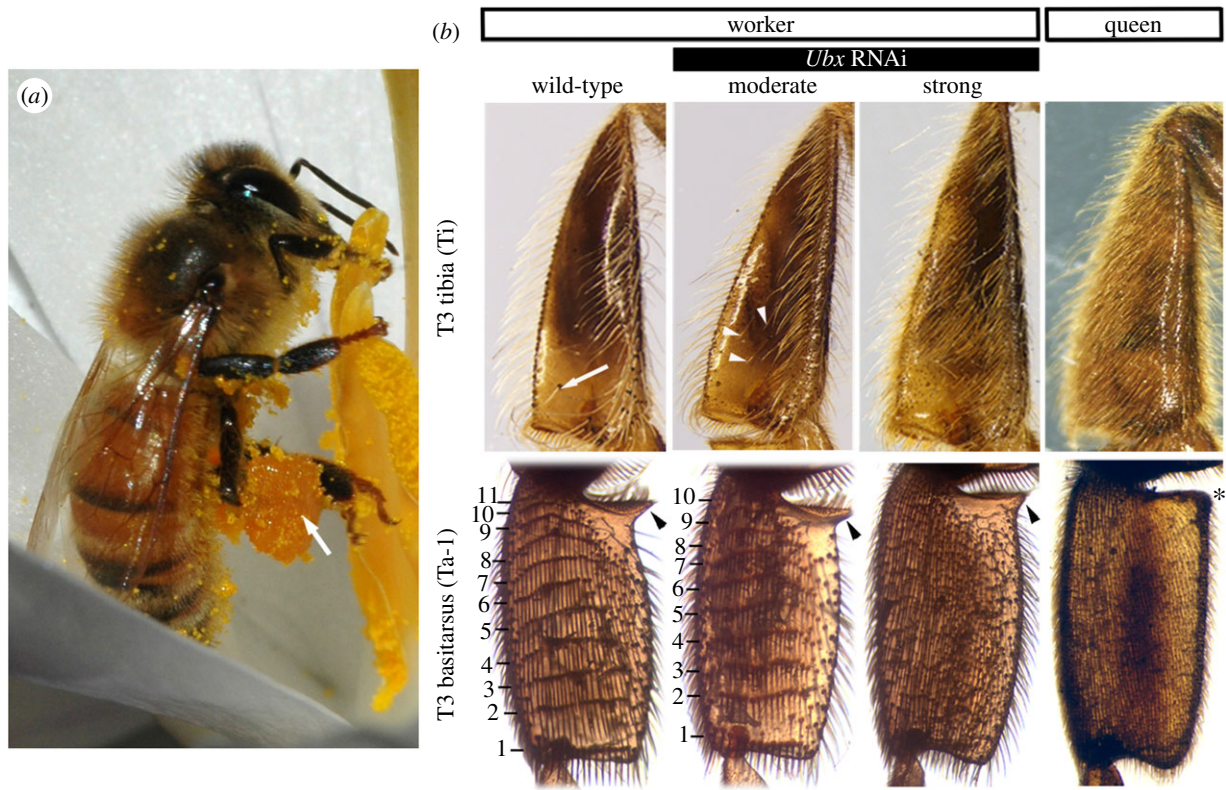


Figure 1. (a) Adult worker bee collecting pollen, the white arrow indicates corbicula filled with pollen granules. (b) Effects of *Ubx*-RNAi on adult worker T3 leg morphology. The white arrow points to the central bristle in wild-type tibia, while white arrowheads point to ectopic bristles in the moderate phenotype. The pollen press on T3 basitarsus is indicated by a black arrowhead in worker bees, while the asterisk denotes the absence of this structure in queens. (Online version in colour.)

to the 5' and 3' ends of the gene were injected independently to control for non-specific or off-target effects. The 309 bp long 5' fragment was amplified with forward 5' ACTCGTATTTGAGCAGACTGCC and reverse 5' GTCGAATACGAGGATGTCGTG primers. For the 3' fragment, the following primers were used: forward 5' ACCATAC GTTCTACCCCTGGATG and reverse 5' AGCAAGTCGAGGAAC TAGCG, generating a 265 bp product. Ten clones of each fragment were recovered, sequenced and compared to each other as well as the existing *Apis Ubx* gene (GenBank accession no. XM623986). The injections were performed on L2 and L3 stages of worker larvae with a Picospritzer II (Parker). Approximately 2 μ l of *Ubx* dsRNA (5 μ g μ l⁻¹) was injected (duration of 60 ms and nitrogen pressure at 20 psi) in five independent trials. We verified the extent of *Ubx* depletion by RT-PCR analysis (electronic supplementary material, figure S1). After injection, the larvae were maintained and reared following an established protocol [11]. Overall, a total of 270 L2–L3 larvae were injected, 38 (14%) of which survived to adulthood. Among the surviving adults, 14 (36%) displayed either a strong or moderate phenotype. These survival rates are in line with similar *Ubx* studies in *Tribolium* in which larval injections caused lethality at the pupal stage, while embryonic injections had 20% survival [5]. In addition, mortality rates of 50% or more have been reported for RNAi experiments in *Apis* [12,13], irrespective of the method used (injections or feeding). To further address non-specific effects, we injected a previously cloned 710 bp fragment of the jellyfish green fluorescent protein [14], and followed the previously described control protocols in *Apis* [15]. A total of 50 L2–L3 stage larvae were injected, of which 15 individuals (30%) survived; all resulting adults showed wild-type phenotype.

(b) Antibody staining

FP6.87 mouse antibody was used to detect the *Ubx* staining in *Bombus* leg discs, as described in Mahfooz *et al.* [6].

3. Results

Honeybee workers transport pollen on the tibial segment of the T3 leg (figure 1a), which is distinguished by having a concave, naked cuticle region that defines the corbicula (figure 1b). Except for a single central bristle (white arrow) thought to stabilize the pollen pellet, this area is completely bristle-free. By contrast, the queen lacks a corbicula and has a tibia that is covered in bristles (figure 1b). The other segment that is involved in pollen gathering is the T3 basitarsus, which is located distal to the tibia. In workers, it features 11 neatly spaced rows of bristles that make up the pollen comb as well as a protrusion known as the pollen press (figure 1b, black arrowhead). Both of these features are absent in queens.

To determine the role of *Ubx* in corbicular development, two non-overlapping *Ubx* fragments were independently injected, yielding similar results; a moderate or strong T3 leg phenotype was observed in 36% of the emerging *Ubx*-RNAi adults (figure 1b). In moderate phenotypes, the tibial segment lost the central bristle while gaining several ectopic bristles (white arrowheads) in the previously naked cuticular area. In strongly affected individuals, the whole corbicular region became completely covered with bristles. Interestingly, the tibia's overall size and its distinct triangular shape remained unchanged indicating that the function of *Ubx* at this developmental stage is primarily in the suppression of bristle development (see electronic supplementary material, figure S2).

Within the T3 basitarsus (figure 1b), the distinct rows of bristles that make up the pollen comb were either reduced in number (moderate phenotype) or they completely lost

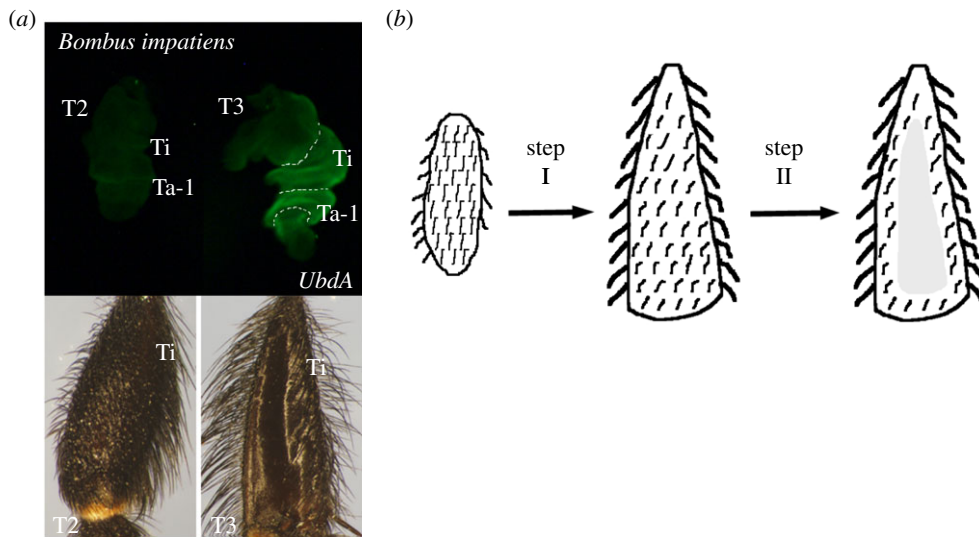


Figure 2. (a) *UbdA* expression in *Bombus impatiens* (bumblebee) leg discs showing expression in the tibia (Ti) and basitarsus (Ta-1) of T3 legs, which corresponds to the presence of a corbicula on the adult T3 leg below. (b) Proposed two-step model for the development of the corbicula. (Online version in colour.)

their organization (strong phenotype). In addition, the pollen press was reduced in size (figure 1*b*, black arrowhead). These combined results in the T3 tibia and basitarsus reveal that *Ubx* plays a critical role in the development of the morphological features that enable pollen collection in *Apis*.

To determine whether this function of *Ubx* may exist in other corbiculate bees, we examined its expression in the leg discs of *B. impatiens*, which belongs to tribe Bombini (bumblebees). In this species as well, the T3 tibia and basitarsus are modified for pollen collection. As shown in figure 2*a*, the *Ubx* expression is restricted solely to these two segments suggesting that *Ubx* may play a general role in the formation of the bee's pollen gathering apparatus.

4. Discussion

The smooth bristle-free surface of corbicula in *Apis* (figure 1*b*) is reminiscent of the 'naked valley' region on the T2 legs in several *Drosophila* species [16]. In the latter instance though, the smooth cuticle is generated by the suppression of the trichomes, which are simple cuticular extensions produced by epidermal cells. However, the honeybee worker pollen basket is generated by inhibiting the development of bristles, which are sensory organs [8]. This suggests that the pollen basket in corbiculate bees results from a novel role of *Ubx* in the suppression of bristles on the T3 tibia. In honeybees, this new role of *Ubx* appears to be under the control of environmental factors (i.e. diet), which leads to varying hormonal levels, resulting in phenotypic plasticity giving rise to the different castes.

Two recent studies [8,17] examined the global changes in expression between *Apis* queens and workers and identified a number of genes that are differentially expressed between the castes. While the functional data are still lacking, some genes, such as *grunge* (*gug*) and *Ataxin-2* (*Atx2*), play a role in the formation of bristles in *Drosophila* [18,19]. As *Ubx* has been shown to regulate the development of specific bristles in the fly thorax [20], *gug* and *Atx2* represent prime candidates as possible *Ubx* targets. Another avenue to explore would be to characterize the role of *Ubx* in

generating distinct T3 basitarsus phenotypes among the castes. As shown in figure 1*b*, while the basitarsus in workers is organized in 11 distinct rows of bristles, in *Ubx*-RNAi adults this pattern is completely disrupted and resembles the situation present in queens. The observed differences in RNAi phenotypes between the T3 tibia and basitarsus suggest the presence of two modes of regulation by *Ubx*. In the former, *Ubx* may have a direct role in bristle suppression (as it is expressed in workers but not in queens). In the latter, the role of *Ubx* in the spatial organization of bristles may be at the downstream target level instead (because its expression in the basitarsus is common to both castes).

As a way of furthering our understanding of the evolution of the corbicula, we propose a two-step process outlined in figure 2*b*. The first step involves the divergence of the T3 tibia from its foreleg counterparts and the establishment of its distinct morphology. This is accomplished by the enlargement of the segment in its distal half and the acquisition of a triangular shape. A similar differential enlargement is also seen in the hind legs of non-social bees suggesting that the T3 enlargement preceded the formation of the corbicula [1]. The second step involves the actual creation of the pollen basket, whose presence is a distinguishing trait between the castes (such as workers and queens). As shown in figure 1*b*, this step is directly regulated by *Ubx* through its repression of bristle development generating a naked cuticular region. The finding that *Ubx* expression in *Bombus* is also restricted to the T3 tibia and basitarsus raises the possibility that this gene may act as a common trigger in the development of the pollen gathering apparatus in corbiculate bees. Further comparative studies in non-corbiculate bees will be necessary to confirm this hypothesis, and in turn will provide insight of how the acquisition of novel functions by *Ubx* may have facilitated the evolution of the social behaviour.

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