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Expression of Hox genes, Zen (GB 51301) ANT and UBX during the development of honey bee (*Apis mellifera)*

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Abstract: Homeobox genes are present in the genome of the honeybee *Apis mellifera* and their expression has been studied principally during embryonic development. The aim of this research was to evaluate expression of three key Hox genes from the larval period to the adult. The expression of honeybee GB51301, Ant and Ubx were examined in 24 and 72 hrs of larvae and pupa, classified on the basis of eye colours, using quantitative RT PCR on either the entire body or the head only. The results show that levels of expression from these genes change in relation to the development of various organs. GB51301(HOX3/zen) expression is mainly localised in the head, and it is expressed in the larvae, when the first nervous cells are formed and in the adult worker. ANTP is expressed in the white-eye pupa at a similar time to the development of antenna. UBX is also expressed in white and red eye pupae at a similar time to leg formation. The expression of these genes is practically absent in the brown eye pupa, when the development of these organs is completed. These genes are found mainly in the body.

Keywords: *Apis mellifera* Honey Bee, Development, Hox Genes, Expression

1. Introduction

Honeybee development consists of four stages: the egg, the larva, the pupa and the adult. The eggs hatch 72 hours after being laid. The pupa stage is divided in other three stages, characterised by the eye colour: white, red and brown [1]. This larval stage lasts for six days, and they then enter the pre-pupa stage. At this time internal changes occur: the white pupa showing the three major body regions similar to those of the adult and the antenna start to develop. After 12 days the eye colour changes and is red by day 15 [1, 2]. During this period the legs develop and complete their structure [3] the brain also completes its development together with the associated ganglia hypo pharyngeal, mandibular salivary glands and antennae [4].

As in other insects and vertebrates, development is, in part, controlled by the expression of a group of regulatory genes: the HOX genes [5]. These genes have well conserved sequences indicating that they were formed before the split between the vertebrates and the insects. Only the class HOX 3 genes represent an exception. Vertebrate and cephalochordate class 3 genes show similar structure with respect to other HOX genes, whereas in *Drosophila*, there is no recognizable class 3 gene. The position between proboscipedia (class 2 homolog) and deformed (class 4) is occupied by three homeobox genes which do not have any homolog in the vertebrates Hox clusters [6]. These genes do not show collinear expression and do not specify axial position during embryogenesis [6]. Two of them, GB51301 [7] and its paralog z2, are expressed specifically in extra embryonic membranes [8]. Hox genes have been especially studied in *Drosophila* where these genes are located in two complexes, the *Antennapedia* [9] and the bithorax complex, which is especially characterised in *Drosophila* [10]. This study was extended to a large variety of Arthropods, between them flies and butterflies [11], with the aim of understanding their role during evolution and the molecular mechanisms that have created diversity between different species of insects.

Of particular interest was the study of these genes in the

honeybee *Apis mellifera* since it is a member of the hymenoptera, a large insect order; *Apis Mellifera* differentiated from *Drosophila* about 250 million years ago. The first Hox genes from a non Drosophilid insect were cloned in the honeybee *Apis mellifera* [12, 13]. *A. mellifera* presents some interesting aspects concerning development, in that there is no involution of the head and more complex behaviour as adults. Many HOX genes have been analysed in the honeybee and their expression was evaluated during the embryogenesis using *in situ* hybridisation [7]. Building on these studies of embryonic development, it is important to understand how the HOX genes are expressed during larval and pupal development, as it is at these stages that the adult structures of the honeybee are developing.

We choose three genes from the HOX complex, localised on chromosome 16, to focus on Zen (Gene GB51301), Antp and Ubx. Different functions are attributed to each of these genes: GB51301 is implicated in the development of nervous system, Antp in the development of Antenna and Ubx in the development of the abdomen and legs [3, 14]. Since different larval stages are characterised by the complete development of these parts, these differences in maturation were examined in order to see if they were accompanied by differential expression of these three genes.

The aim of the study is therefore the analysis of expression of these three genes in relation to the development of *Apis Mellifera* head, antenna and legs.

2. Materials and Methods

2.1. Beekeeping

The honeybee colonies were cultured using standard techniques in Italy. Honeybee embryos were collected from frames removed from nucleus boxes containing small honeybee colonies. *Apis mellifera* workers, derived from Ligustica specie, of different ages were collected from colonies held by Prof Gardi (University of Perugia, Italy).

Developmental stages used were larvae of 24 and 72 hrs, and pupae distinguished on the basis of eye colour [1, 15]:

- White-eyed pupae – WEP White eyes and white body
- Red-eyed pupae – REP Red eyes and white body
- Brown-eyed pupae – BEP Brown eyes and white body
- Adult worker honeybee.

The insects were immediately frozen in liquid nitrogen and maintained at -80 C°.

Analysis of gene expression in pupae and adults was made on either the entire insect, or on the separated head only (in this case, proboscis and antennae were removed). Two biological replicates were run in three technical replicates for twice.

2.2. RNA extraction

The frozen tissue was weighed and the RNA was extracted with the Norgen's Total RNA Purification kit (Norgen Biotek Corp.Ontario, Canada), according to the manufacturer's instructions. The isolated RNA was evaluated with a spectrophotometer, and RNA integrity was assessed using 1.2% agarose gel electrophoresis.

Reverse Transcription was used, and was completed by employing the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA). The program of the thermal cycler (MyCycler™, Bio-Rad, Hercules, CA, USA) provided: step 1, 25°C for 10 min, step 2, 37°C for 120 min, step 3, 85°C for 5 min.

2.3. RT-PCR

Quantitative RT-PCR was performed using an Applied Biosystems 7.300 RT-PCR machine and Master Mix TaqMan®Gene Expression (Applied Biosystems, Foster City, California, USA). Thermocycling conditions were: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds and 60°C for 1 minute for 40 cycles.

Relative Quantification: this method describes a RT-PCR experiment in which the expression of a gene of interest in one sample (i.e. treated) is compared to the expression of the same in another sample (i.e. untreated). The Delta-delta Ct values comparison method was used [16]. The results are expressed as fold change (increase or decrease) in expression of the treated in relation to the untreated sample. A normalized gene is used as a control for experimental variability in this type of quantification. Fold increase above 1 indicated genes over expression and fold decrease under 1 indicated genes down regulation. A relative quantity of 1 indicates no change in expression level.

2.4. Genes

GB50986-RA was the best gene for normalizing gene transcription data and was used in our work, since it was stable during the analysis (Table 1).

Table 1. *Primers (forward and Reverse) and Probe for used genes*

	Primers Forward	**Primers Reverse**	**Probe (TaqMan MGB):**
GB50986-RA (normalizing gene)	CTT CGA ATC ACG TCG GTT TGT	TTG ATC GCG GCG TTC AT	CGC GAT GAA GAA CGG
GB 51301 Gene	GGA CAA CAG CGT GGA TGG A	CAC CGG CCT CTG CAT CTC	CGT GA CGA ATT TAC GAG G
ANTP Gene	ACC CGT GGA TGA GAA GTC AAT T	TTT GGT ATC GGG TGT ACG TTT G	AGA GGA AAC GAG GCC G
UBX Gene	ATC AGC AAC CCA GCA ACC ATA C	CAT TCC GTT CGC TCC TGC TA	TTC TAC CCC TGG ATG GC

Each of the two biological samples was run in three technical replicates, with the aim to avoid the differences between the two examined samples (differences were less than $p<0.05$, t-Student).

3. Results

The quantitative expression of the genes GB51301, ANTP and UBX was evaluated at different stages of *A. mellifera* larval and pupal development. Using as reference the expression of the adult, GB51301 showed strong expression, six times that of the adult in the larva at 24 hrs, decreasing at 72 hrs to reach almost the value of the adult (Fig.1, Table 2).

The pupal stage expression was four times that of the adult in WEP decreasing to a value less than the adult and disappearing completely in REP (Table 2).

Table 2. *Relative expression of the genes GB 51301, ANTP and UBX in larvae and pupa body of Apis mellifera. The values are compared to adult values which are considered equal to 1.*

	Expression Fold Change					
	Adult Bee	**Larva 24 h**	**Larva 72 h**	**WEP Body**	**REP Body**	**BEP Body**
GB51301 Gene	1	5.90 ± 1.66	1.42 ± 0.36	3.99 ± 0.34	0.47± 0.1	0.009 ± 0.02
ANTP gene	1	1.3 ± 0.7	1.4 ± 0.8	9.71 ± 1.7	6 ± 2	0.25 ± 0.38
UBX gene	1	10 ± 4	9 ± 2	50.7 ± 1.3	19 ± 6	0.45 ± 1

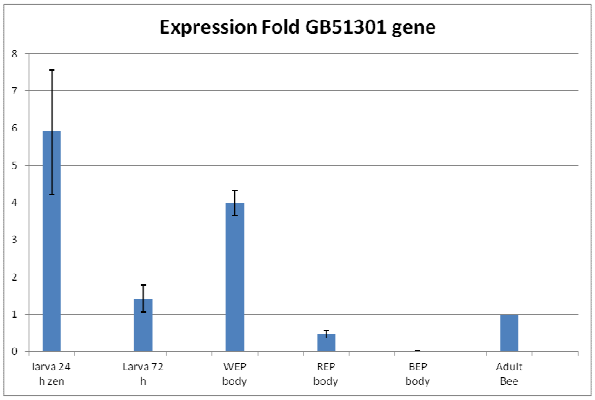

Figure 1. *GB51301 gene, compared with expression in the adult. Y Axis denotes the expression relative to the adult, equal to 1.*

This pattern of expression is different to ANTP expression. For this gene, the expression levels in the larval period are similar to that of the adult, followed by a strong increase in WEP and a smaller increase in REP while in BEP decreases to less than the adult (Fig. 2, Table 2).

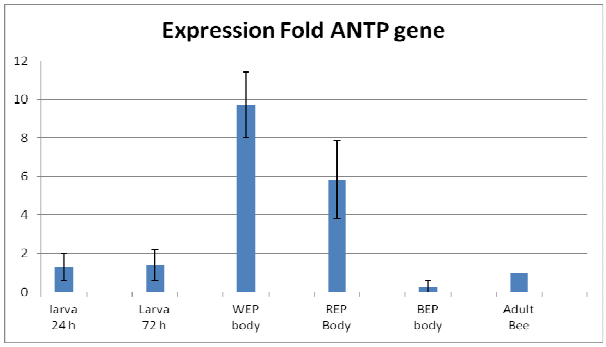

Figure 2. *ANTP gene, using the expression of the adult as reference. Y Axis denotes expression relative to adult.*

The gene UBX is practically unexpressed in adult and BEP pupa. The level of expression increases somewhat in the larvae of 24 and 72 hours. Highest expression can be seen in WEP followed by a lower, but still high value, in REP (Fig.3 Table 2).

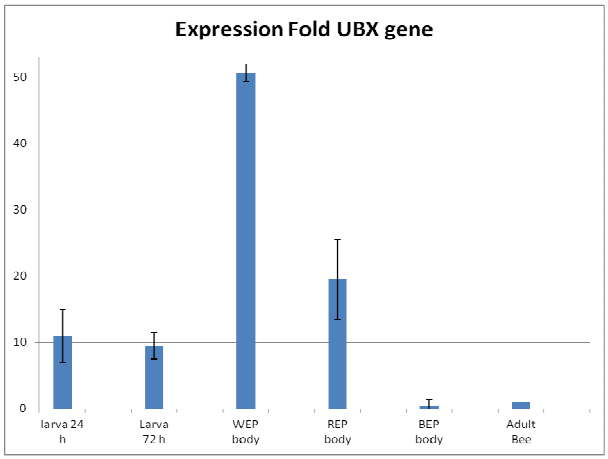

Figure 3. *UBX relative gene expression. Y Axis denotes expression relative to adult.*

The analysis of the expression in the isolated brain show that GB51301 is expressed at levels higher than adult in the 24 hrs larvae, then decreases at 72 hrs to a value less than that of the adult through a small increase is observed in the WEP, but with half value of that of the adult; there is a decrease in REP and low expression in BEP (Fig. 4, Table 3).

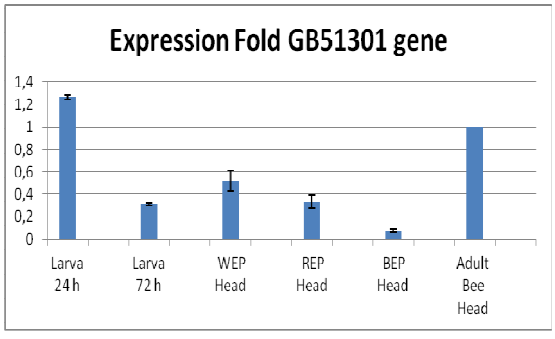

Figure 4. *GB51301 gene, analysis of the expression in the head. Y Axis denotes expression relative to adult.*

Table 3. *Relative expression of the genes GB 51301, ANTP and UBX in larvae and pupa head of Apis mellifera. The values are referred to adult values which are considered equal to 1.*

	Expression Fold Change					
	Adult Bee Brain	**Larva 24 h**	**Larva 72 h**	**WEP Head**	**REP Head**	**BEP Head**
GB51301 Gene	1	1.27 ± 0.02	0.31 ± 0.01	0.52 ± 0.09	0.33 ± 0.06	0.07 ± 0.01
ANTP gene	1	3.81 ± 0.01	4.12 ± 0.01	1.37 ± 0.3	2.4 ± 0.6	0.7 ± 0.2
UBX gene	1	195 ± 1	169 ± 2	22 ± 6	33.5 ± 0,1	1.69 ± 0.5

If these values are compared with those obtained for the entire insect it is possible to see that the 24 hrs larval expression is six times that of the adult, it decreases at 72 hrs, increases again in WEP and a small expression is observed in BEP. In the head instead the value remains almost the same in WEP and REP, suggesting that this gene is expressed mainly in the nervous system.

ANTP is expressed at lower levels in the head of adult and BEP compared to larvae of 24 and 72 hrs in which the values are almost 4 times that of the adult. In WEP and REP the values are 1.5 and 2 times respectively that of adult (Fig. 5, Table 3).

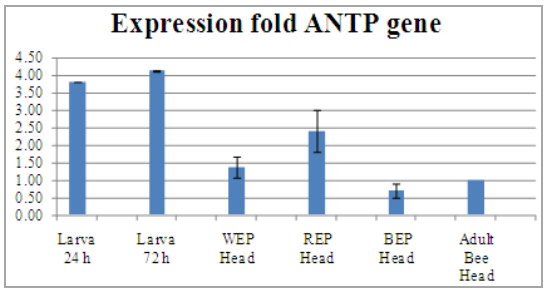

Figure 5. *ANTP gene, analysis of the expression in the head. Y Axis denotes expression relative to adult.*

The difference between total body and head expression suggests that this gene has low expression in the nervous system. In fact, the expression of this gene in the 24 hrs larvae is similar to that of the adult, whereas in the head the values are 4 times higher. Also in the WEP the values on the entire insects are ten times that of the adult; instead, in the head there is only 1.5 times and the decrease observed between WEP and REP is not evident in the head. It is evident that this gene is only partially expressed in the head and that more intensive expression is in the WEP and REP.

The gene UBX is practically unexpressed in the adult and BEP head while a small increase is observed especially in the REP, in coincidence with legs development (Fig.6, Table 3).

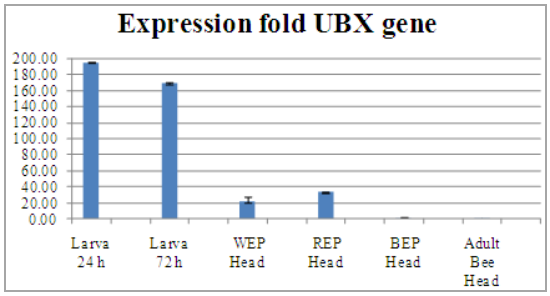

Figure 6. *UBX gene, analysis of the expression in the head. Y Axis denotes expression relative to adult.*

In conclusion, the three HOX genes examined showed variations in expression during the development of *A. mellifera* and present different localisations: GB51301 is mainly expressed in the head, whereas the other two are expressed in the body; they are less involved in the larval period, but principally in the pupal stage of WEP and REP.

4. Discussion

HOX genes control the expression of many other genes and are involved in the structural organisation of organisms. The HOX complex in the Honeybee is large with respect to other insects mainly due to intragenic regions. The gene GB51301, identified as HOX3/zen, is the only one in the honeybee and is expressed in the extra-embryonic membranes of the early bee embryos. In the late honeybee embryo GB51301 is expressed also in the nervous system [7]. The other two HOX genes examined here are conserved during evolution. The pupal stage in the honeybee is long with respect to other insects and to the embryonic development [4]. An increase in body weight is observed in relation to honey production as well an increase in head weight with higher royal jelly production [17]. The head weight is higher in 13 day old pupae and maintains the same value until 17 days corresponding to the dark eye [1], then it decreases reaching a minimum at 20 days [4]. Proteomic analyses also show that young pupal head requires specific proteins to coordinate the development of specific organs. The brain structures are practically absent from the larvae and develop during the pupal period. This development is due to formation of new neurons and the death of others with a different intensity between the queens, workers and drones [15]. As a consequence, the development of many organs like antennae and legs happens during the pupal period [18, 19]. The expression of the HOX genes studied, GB51301, ANTP, UBX, presents different intensities with respect to age. In the 24h larvae, GB51301 is intensively expressed with respect to the adult, whereas ANTP and UBX do not. At 72 hrs, larval expression of GB51301 decreases with a second increase occurring in WEP.

Comparing these results with those obtained on analysing only the pupal and adult heads, the expression in the adult heads of workers is only a little less with respect to the 24 hrs larvae when the formation of the adult nervous system starts. The gene expression is in agreement with the development of neuronal circuits in relation to the complex behaviour of the workers including the development of memory [15]. Farris et al. [19] have demonstrated an increased volume of neuropil associated with the mushroom bodies, a brain region involved in learning, memory, and sensory

integration. This increase in volume of neuropil is due to the development of dendritic processes. The expression of ANTP and UBX in the larvae was described with particular reference to their localisation and changes with time. ANTP appeared in the 32hr old embryos and is localised principally in the ventral part, later diffusing to the dorsal [20]. In 40 hrs embryos, a strong expression is seen in the second thoracic segment. Later the expression appears in the areas around the abdominal pits together with UBX, indicating a role in tracheal development [20]. Our quantitative measurements of the expression in the larvae are similar to the adult. It is known that the antenna develop in the pupa when ANTP expression is high, especially in WEP [19]. The values are again low in BEP when the antennas are completely developed. The role of this gene in antenna formation may therefore be proposed. As regards the localisation at level of head, it is very weak suggesting that the gene expression in the pupae is mainly in the body. UBX is also expressed in the red and white eyed pupae a period in which the morphogenesis of the legs is completed.

Also, if the phenotype of apex is determined during the four and five days of the larval stage, these genes are expressed intensively only in the pupal period and the expression is completed in BEP. The expression of UBX gene paralleled the leg development and it is localised mainly in the body since only a very small expression is observed in the head. In fact it appeared to be localised in tibia basitarsu of adult legs [3].

Medved et al. [13] proposed that the development of corbicula may be happens through two-step process: one during larval period and a second step which involves the creation of pollen basket with distinction between the casts.

This hypothesis is confirmed by our results showing an increase of expression of UBX which is very high in the WEP.

In conclusion with this analysis we have demonstrated that the values of expression of the HOX genes changes during the development of *A. Mellifera*. We have also shown that GB51301 is mainly localised in the head and it is expressed also in the adult thus contributing to the complex function of the nervous system in the workers. The influence of environmental factors on the expression of these genes may help to understand better their role.

5. Conclusion

Three Homeobox genes expression has been studied in *Apis Mellifera* during the development The genes are GB51301, ANTP, UBX localised respectively in the nervous system, thorax and abdomen. Using quantitative PCR, the expression in the larval and pupal period changes in relation to the development of specific organs like antenna and corbicula. The GB513011 localised in the nervous system maintains high value of expression also in adult confirming its role in contributing to the nervous system complex functions in adult worker.

The study of Homeobox genes in different life period may help in monitoring different phases of *Apis Mellifera* development.

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