Comparative Analysis of Sexual Dimorphic Ecdysteroid Regulating Mblk & E74 Gene Expression Levels by Real Time PCR from *Galleria mellonella*

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Authors' contributions

This work was carried out in collaboration between all authors. Author SPS designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors SS and ISS managed the analyses of the study. Author SR managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

The Greater wax moth *Galleria mellonella* belongs to the family of Pyralidae which cause great damage to the honey bee yield worldwide, a major insect model. The honey bee cultivation places are most habitat of this insect. Most the organisms of insects rely on their olfactory system to detect and analyse the sensible nature in the environment and it is mostly useful in the concept of behavioural emotions includes mating, host defence and other sensory related molecular functions. This organism is the only member of the genus *Galleria* and also it is found in most the places in the world. The larvae of *Galleria mellonella* is used as commercial product as proving the food for predatory insects. The pathogenicity and toxicology testing were making the researchers easy by replacing the use of small mammals for in-vivo and in-vitro studies. The neurological mechanism of this insect is still unclear.

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The ecdysone induced protein is unique protein with various functions, types, diversity in different organisms. From the previous study indicate that the ecdysteroid regulated gene - E74 & mushroom like large body specific protein expressed in kenyon cells of brain and was involved in olfactory, neurological behaviour of insects, embryonic and larval development. The gene expression pattern was predicted by using real time PCR. From the analyzed results, that the genes MBLK & E74 genes were expressed in *Galleria mellonella* which allow us to better understand of neurological disorders by using this organism as a conventional animal model.

1. INTRODUCTION

A pest of unprotected honey bee combs in weakened colonies or neglected hives. "As the larvae chew through the comb, they spin a silk-lined tunnel through the cell walls and over the face of the comb. These silk threads can tether emerging bees by their abdomens to their cells and they die of starvation because they are unable to escape from their cell. This phenomenon is termed galleriasis" [U. of Georgia]. The larvae are used as experimental subjects in insect physiology labs, as fish bait, and in the study of nematodes.

Recently it has been discovered that the larvae can digest polyethylene, reducing it to ethylene glycol [1]. This may be very valuable to control plastic pollution.

The recent researches at the University of Strathclyde have discovered that the *Galleria mellonella* is having the ability to sensing the sound wave frequencies range up to 300 kilo hertz (kHz) is considered as the highest analysed sound frequency sensing organism in the world creatures. Humans have hearing sound sensing up to20 kHz maximum, minimum to around 12-15 kHz as they get age, and most dolphins sensing limitations around 160 kHz.

Dr James Windmill, who has led the research at Strathclyde, said: "We are extremely surprised to find that the moth is capable of hearing sound frequencies at this level and we hope to use the findings to better understand air-coupled ultrasound". "The use of ultrasound in air is extremely difficult as such high frequency signals are quickly weakened in air. Other animals such as bats are known to use ultrasound to communicate and now it is clear that moths are capable of even more advanced use of sound. Dr Windmill's multi-disciplinary research team is now working to apply the biological study of this,

and other insect ears to the design of micro-scale acoustic systems. It is hoped that by studying the unprecedented capabilities of the moth's ear, the team can produce new technological innovations, such as miniature microphones [2].

Host selection by female moths is fundamental to the survival of their larvae. Detecting and perceiving the non-volatile chemicals of the plant surface involved in gustatory detection determine the host preference. In many lepidopteran species, tarsal chemosensilla are sensitive to non-volatile chemicals and responsible for taste detection. These wax moths are reared commercially as feed for predatory insects. The main advantage in rearing of these wax moths includes no special dietary supplements, odourless, safe handling, non-pathogenic to humans, easy to maintain the culture and this would not fool the researches from being grow once it reaches the larval stage [3].

2. ECDYSTEROID REGULATED GENES

During insect oogenesis, the oocyte acquires nutrients and genetic determinants to support embryonic development (previtellogenesis and vitellogenesis) and subsequently becomes surrounded by a protective eggshell (choriogenesis). In many insects, ecdysteroids are synthesized during ovarian growth which is often followed by the accumulation of ecdysteroid conjugates into the eggs. The exact role of the ecdysteroids during oogenesis remains largely unclarified although functions as paracrine or autocrine regulators to signal the progression of follicle development of the resumption of meiosis in the oocyte have been proposed.

Genetic studies also established that progression of oogenesis in Drosophila requires the function of genesimplicated in the ecdysone regulatory hierarchy such as EcR, BR-C and E75 [4] as well as genes involved in synthesis of

ecdysteroids. These research works suggest that the ecdysteroid regulated genes are involved in the oogenesis developmental stage in insects.

2.1 Mblk Gene

Mushroom bodies (MBs) are considered to be involved in higher-order sensory processing in the insect brain [5]. Proteins with high homology in the DNA binding motifs of Mblk-1 are found widely in the animal kingdom [6]. Mushroom bodies (MBs)[1] are believed to be involved in sensory integration, learning, and memory in insects [7,8]. The honeybee MBs are well developed when compared with those of other insects. In the honeybee, the ratio of volume of MBs to that of whole brain is ~12%, whereas that of Drosophilais ~2% [5]. Moreover, each MB of the honeybee has two calyces composed of two morphologically distinct types of interneurons, the large- and small-type Kenyon cells [9-11]. On the other hand, in Drosophila, there is only one calyx, and the Kenyon cells are morphologically indistinct [12]. These observations suggest that MB function is closely associated with the advanced honeybee behaviors.

In this study we are using RT-PCR to analyse the sexual dimorphic gene expression level of E74 & Mblk of greater wax moth. In case of gene expression studies, Brunner and coauthors [13] reported that not all of the best known reference genes are equal. To date, there are many studies in which the search and validation of reference genes are reported, but most of them are focusing on the traditionaly used "house keeping" genes, not novel candidate reference genes that have been inferred from genome-wide studies such as in [13].

3. EXPERIMENTAL METHODOLOGY

3.1 Ethical Statement

The Greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralid Moths) is a common honey comb pest, the infected honey comb collections were made with the direct of Sharmee bee farm (7/96, Mudangiar Road, Near Raju's College, Rajapalayam – 626 117, Viruthunagar District). It's not included in the "List of Endangered and Protected Animals in India". All operations and handling of moths were performed according to ethical guidelines in order to minimize pain and discomfort to the moths.

3.2 Insect Rearing

Galleria mellonella larvae were originally collected from registered honey bee farm at Sharmee bee farm (7/96, Mudangiar Road, Near Raju's College, Rajapalayam – 626 117, Viruthunagar District, India. Larvae stage wax moths were maintained under the room temperature (37°C) in day and light photoperiods.

3.3 Sample Collection

For gene expression analysis, approximately 120 adult wax moths of male and female individuals were collected. The live wax moths were killed by using insect killing jar followed entomological protocol. Then immediately the adult wax moths were immersed in liquid nitrogen and it was stored at -80°C till the total RNA isolation. All the experimental works are followed by aseptic conditions.

3.4 Total Rna Isolation

The total RNA was isolated from male and female wax moths by using MACHEREY-NAGEL Nucleospin@ RNA kit followed by manufacturer's instructions. The quality, concentration and integrity of isolated RNA was analysed by using UV spectrometer. The quantification of total RNA was determined followed by the agarose gel electrophoresis.

3.5 cDNA Synthesis and Library Construction

The relative expression of E74 & mblk genes was identified by Real-Time PCR of Transcriptor first strand cDNA synthesis, Roche, Switzerland. The template- primer mixture (20μl) which contains total RNA 8μl; Oligo dT 1μl; Water 4μl; RT reaction buffer 4μl; Protector RNase inhibitor 0.5μl; Deoxynuclotide mix 10mM 2μl; Reverse Transcriptase 0.5 μl was prepared. The template primer mixture was denatured by heating the tube for 10min at 65°C in the thermal block cycler with a heated lid (to minimize evaporation). The tube was cooled with ice and the remaining components were added to the mix. After adding all components, the reagents were mixed in the tube gently and incubated the RT reaction for 60min at 50°C. Reverse transcriptase inactivated by heating to 85°C for 5 min. The reaction was terminated by placing the tube on ice.
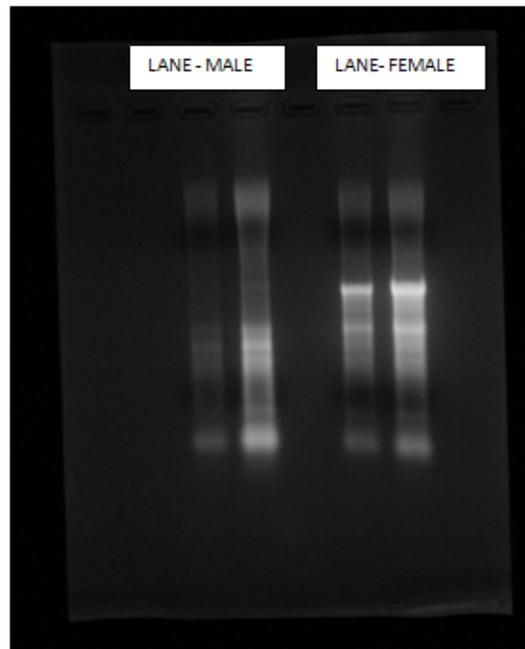
Fig. 1. The Lane 1 represents the male RNA band and Lane 2 represents the female RNA band which is isolated in an aseptic condition

3.6 RT-PCR Analysis

The expression of E74 gene in greater wax moth, *Galleria mellonella* were evaluated by RT-PCR. In briefly the primers used for cDNA synthesis are Negative Control (GAPDH) were used as reference gene and the target genes E74 Primer with the sequence of Glyceraldehyde 3-phosphate
dehydrogenase (GAPDH, XM_393605.5), 5'-GATGCACCCATGTTTGTTTG-3'/ 5'-TTTGCAGAAGGTGCATCAAC-3' (reference gene), E74 – 5' -GGCAACTGTGCTGTGGTAGCTGT -3' /
5' -CCGTAGTATAGCTCGCCTCTCGT - 3' and Mblk-1, 5'-CAACACCAAATACGACCCAAAAC-3' and 5'-GACAACAGCGGCTTCAAC-3'

3.7 RT-PCR Conditions and Preparations

Preparation of Master Mix was followed by thaw one vial of Reaction Mix and shield it from light. Briefly centrifuge one vial enzyme and the thawed vial of reaction mix and pipette 10µl from vial into another vial. Mix gently by pipetting up and down. Do not vortex. Preparation of PCR mix was followed by using depending on the total number of reactions; place the required number of LightCycler Capillaries in precooled centrifuge adapters or in a LightCycler Sample Carousel in a precooled LightCycler Centrifuge Bucket. Prepare a 10X conc. Solution of the PCR primers. In a 1.5ml reaction tube on ice, prepare the PCR mix for one 20µl reaction by adding the following components in the order mentioned are as follows; Water PCR Grade , MgCl2 stock solution, PCR Primer, 10X concentration - 2µl, LightCycler FastStart DNA Master SYBR Green I, 10X concentration - 2µl, Total Volume - 18µl. Mix carefully by pipetting up and down. Do not vortex. Pipet 18µl PCR mix into each precooled LightCycler Capillary. Add 2µl of the DNA template. Seal each capillary with a stopper. Place the adaptors with capillaries into standard benchtop microcentrifuge. Centrifuge at 700xg for5s. Transfer the capillaries into the sample carousel of the LightCycler instrument.

3.8 Gene Expression Analysis

Obtained Ct values from male and female moths sample separately comparing with reference gene (GAPDH) and its amplification efficiency is 2.0. The relative gene expression levels were calculated by using $\Delta\Delta$ Ct method. These genes were widely expressed in many insects kenyon cells of brain which is associated with the neurological function according to a genome-wide scientific studies.
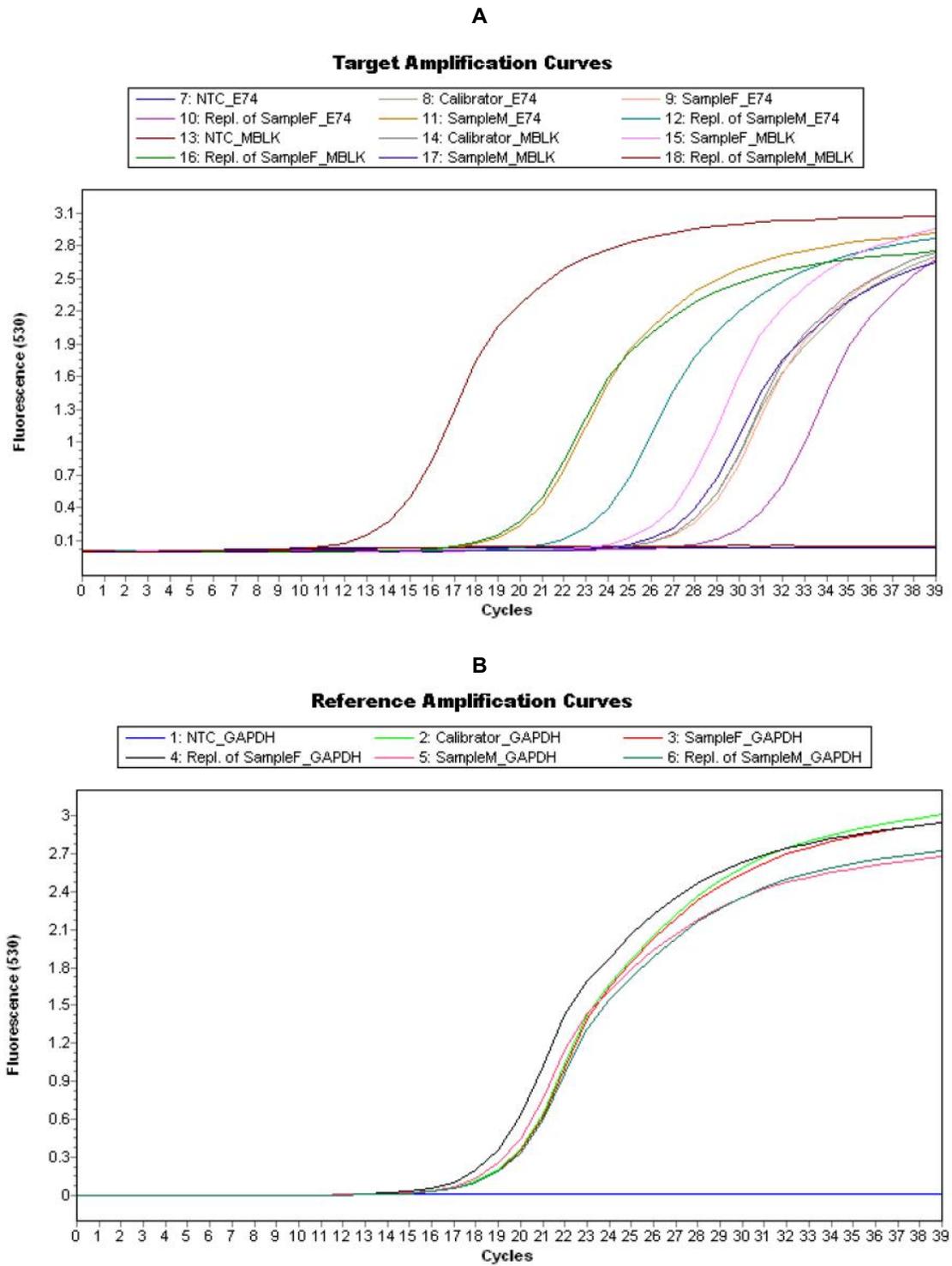
4. RESULTS

A

B

Fig. 2. The A graph represents the Target genes Amplification curves (E74, MBLK of male and female genes separately and B graph represents the Reference gene GAPDH.

A
Melting Curves

1: NTC_GAPDH	2: Calibrator_GAPDH	3: SampleF_GAPDH
4: Repl. of SampleF_GAPDH	5: SampleM_GAPDH	6: Repl. of SampleM_GAPDH
7: NTC_E74	8: Calibrator_E74	9: SampleF_E74
10: Repl. of SampleF_E74	11: SampleM_E74	12: Repl. of SampleM_E74
13: NTC_MBLK	14: Calibrator_MBLK	15: SampleF_MBLK
16: Repl. of SampleF_MBLK	17: SampleM_MBLK	18: Repl. of SampleM_MBLK

B
Melting Peaks

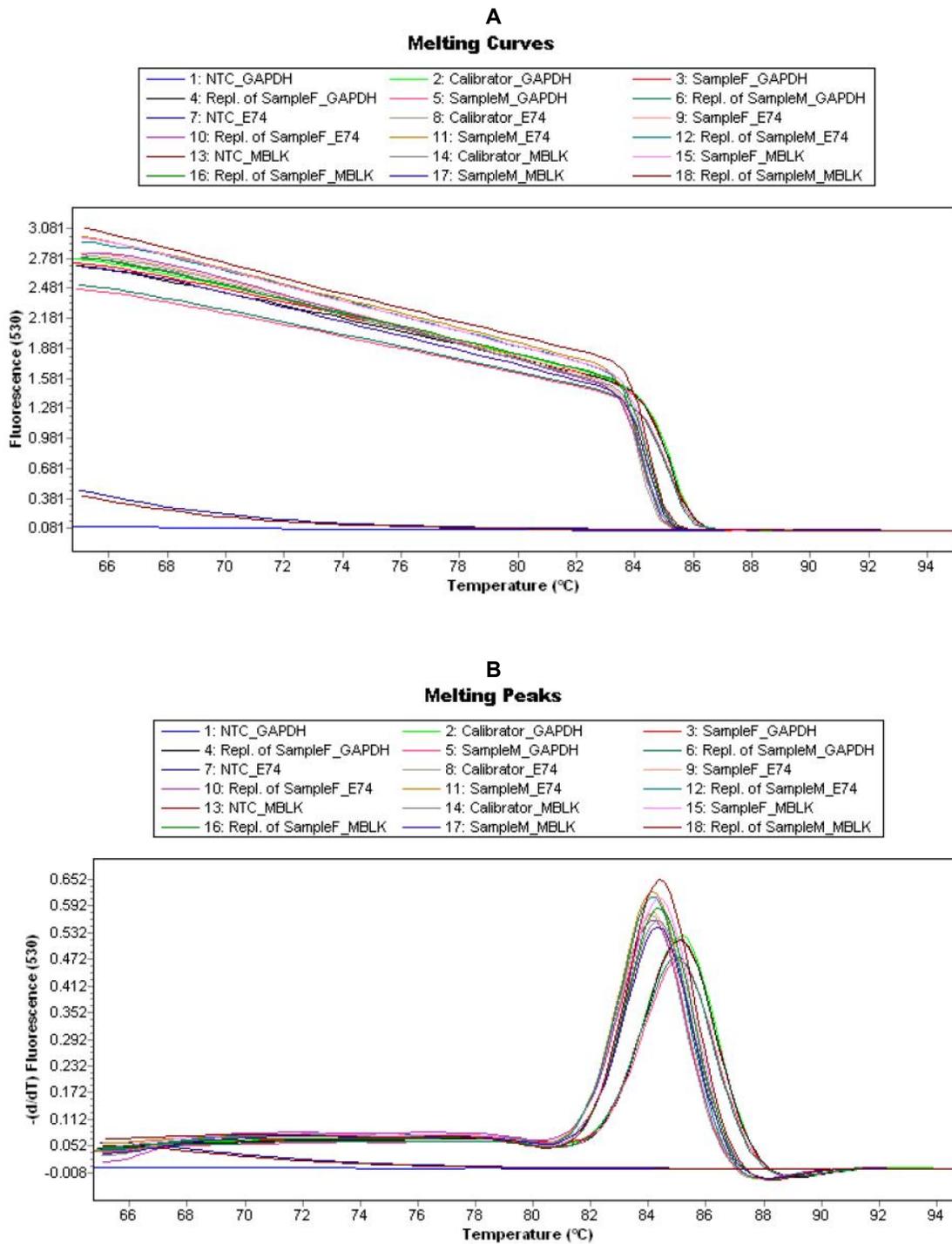|		
1: NTC_GAPDH	2: Calibrator_GAPDH	3: SampleF_GAPDH
4: Repl. of SampleF_GAPDH	5: SampleM_GAPDH	6: Repl. of SampleM_GAPDH
7: NTC_E74	8: Calibrator_E74	9: SampleF_E74
10: Repl. of SampleF_E74	11: SampleM_E74	12: Repl. of SampleM_E74
13: NTC_MBLK	14: Calibrator_MBLK	15: SampleF_MBLK
16: Repl. of SampleF_MBLK	17: SampleM_MBLK	18: Repl. of SampleM_MBLK

Fig. 3. The A graph represents the melting curves for E74, MBLK of male and female and GAPDH (Reference gene) and B graph represents the melting peaks for E74, MBLK of male and female and GAPDH (Reference gene)
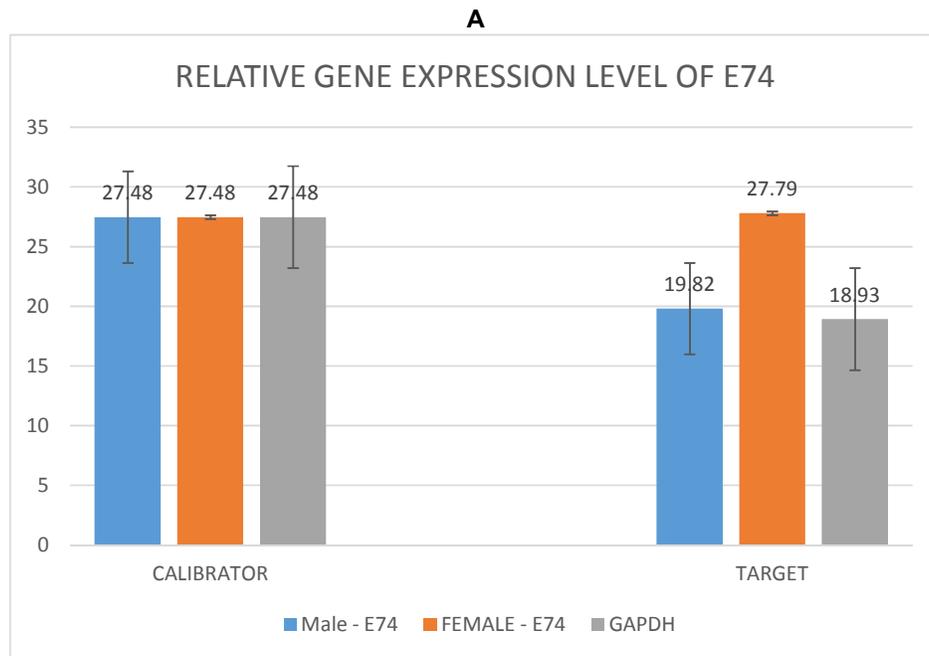
A

B

Fig. 4. The relative gene expression level of E74 & MBLK genes with the given expression level values for calibrator, target and reference gene expression levels. The error bar represents the standard error of the mean. The values indicating the gene expression levels of tested (E74 & MBLK) and reference gene (GAPDH)

5. DISCUSSION

From the past 2 decades, many aspects there was an uncovered information and in particular, the underlying mechanism of brain functions in many insects and the neurological associated genes which control regulation of neuro-circuits of insects. The expression and interaction of these mushroom body genes with other genes revealed the breakthrough in neurogenetics studies. The honeybee, fly (Drosophila), cricket, grasshopper, locust, and cockroach (Periplaneta) dominate the literature, but valuable generalizations and comparative considerations can be drawn from work on other species [14]. The mushroom bodies (corpora pedunculata) of the insect brain are a pair of easily discernible neuropils comprising thousands of densely packed parallel neurons running on either side of the central complex from back to front and downward through the protocerebrum [15]. They consist (from back to front) of the rind of Kenyon cell bodies followed by an often cup-shaped protrusion called the calyx, stalk, or peduncle and finally two lobes pointing in roughly orthogonal directions (vertical and medial). The MB neuropil is separated from the rest of the brain by a thin sheath of glia lamellae. The Kenyon cells, also called intrinsic neurons, stay within this sheath. Their arborizations constitute its overall shape and they contribute the bulk of the MB neuropil. Kenyon cell bodies send thin fibres to the calyces. These fibers then give rise to the dendrites that make up the calyx neuropil. Each dendritic tree provides an axon into the pedunculus. Kenyon cell dendrites are postsynaptic to afferents reaching the calyces and the axons are interconnected by occasional synapses. At the ventrorostral end of the peduncle the fiber bifurcates, one branch growing upward into the (vertical) α-lobe, the other into the (medial) β-lobe toward the mid-plane. Although their location, small cell body size, and arborization pattern makes Kenyon cells easy to recognize as a distinct class of cells, several subtypes can be distinguished by genetic [16].

The Kenyon cells are only two synapses away from the olfactory receptors on the antenna. The simple, anatomy-based assumption that the glomeruli of the antennal lobe receive receptor fibers of the same chemospecificity and that the projection neurons relay this information from each glomerulus to the calyx is certainly a simplification but it may still embody some important properties of the olfactory input to the MB. As the anatomy suggests, a limited number of projection neurons with distinct chemospecificities carry the information that enables the animal to discriminate thousands of odors. This can only be achieved by evaluating the activity of the projection neurons in a combinatorial manner. The divergence from the projection neurons to the Kenyon cells mentioned above may reflect such a combinatorial principle [17-18].

From the comparative studies of mushroom bodies in different insects have revealed about Mbs function in optic lobes, olfaction functions and some lepidoptera species like *Galleria mellonella* organism complex lobe functions have to find in future studies.

The MBs of Drosophila and the honeybee are involved in certain forms of olfactory conditioning. How they contribute to the behavioral plasticity is less well understood. In flies, cAMP signaling is required in the Kenyon cells, suggesting that synaptic transmission between Kenyon cells and extrinsic output neurons is modified by the respective reinforcer. Whether the synaptic plasticity in the Kenyon cells is sufficient to provide olfactory memory remains an open question [19].

The two ecdysteroid-regulated genes, *Mblk-1* and *E74*, are expressed selectively in Kenyon cell subtypes in the mushroom bodies of the honeybee (*Apis mellifera* L.) brain [20]. A putative DNA binding motif of Mblk-1 had significant sequence homology with those encoded by genes from various animal species, suggesting that the functions of these proteins in neural cells are conserved among the animal kingdom [21]. The steroid hormone ecdysone initiates Drosophila metamorphosis by reprogramming gene expression during late larval and prepupal development. The ecdysone-inducible gene E74, a member of the ets proto-oncogene family, has been proposed to play a key role in this process [22]. The E74 transcripts are also widely expressed as metamorphosis begins. E74 early genes are induced directly by ecdysone in overlapping temporal and spatial patterns during the onset of Drosophila metamorphosis [23].

6. CONCLUSION

From this gene expression studies, these two ecdysteroid regulating genes (E74 & MBLK) homolog of drosophila ecdysteroid regulating

genes are also expressed in *Galleria mellonella* genomic DNA by using Real Time PCR analysis was demonstrated that the gene E74 get highly expressed in female moth and the gene mblk was highly expressed in male moth. From the gene expression level indicates that the two ecdysteroid regulating genes were got differently expressed level with sexual dimorphic conditions of greater wax moth. Need to work more on this tissue specific gene expression patterns of mushroom bodies of kenyon cells of *Galleria mellonella* in my future research work.

SUPPORTING INFORMATION

Real Time - PCR parameters and conditions. Melting curves & peaks generated for all genes along with NTC (Negative Control).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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