International Congress Series 1288 (2006) 861–863

www.ics-elsevier.com

# Estimating the post-mortem interval (II): The use of differential temporal gene expression to determine the age of blowfly pupae

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**Abstract.** To establish the minimum post-mortem interval (PMI) from insect evidence, an entomologist needs to accurately determine the age of the insects present on a discovered body. The aim of this work was to establish whether temporally expressed genes could be utilised as age markers for immature Dipteran insects. This study focused upon the pupal stage of *Calliphora vicina* (Diptera: Calliphoridae). Differentially expressed genes were located by differential display or from previous research. The expression of these genes during the pupal stage was quantified using real-time PCR. This preliminary research indicated that measuring gene expression is a viable technique to establish the age of immature *Calliphora* insects. 

*Keywords:* *Calliphora vicina*; Age determination; Pupal stage; Post-mortem interval; Forensic entomology

## 1. Introduction

Insect evidence can be utilised in a forensic investigation in a variety of ways. For instance, insects are most commonly used to help in the estimation of time since death of a discovered corpse. To establish time since death, an entomologist requires accurate assessment of the age of insects discovered associated with a corpse and hence the minimum post-mortem interval (PMI). At present this is done using morphological features or biometric characteristics such as length or weight [1]. The aim of this work was to use molecular techniques to determine the age of immature forms of forensically

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0531-5131/ 
doi:10.1016/j.ics.2005.09.091

important fly species. Throughout the developmental lifecycle of insects different genes will be expressed at specific time points. Once identified these temporally expressed genes could provide markers as to the age of an insect.

## 2. Methodology

### *2.1. Samples*

Wild populations of *Calliphora vicina* (Diptera: Calliphoridae) were maintained in the laboratory. This species is an early corpse invader in the United Kingdom. Initially the pupal stage would be focused upon. Adult females were encouraged to lay eggs and this timepoint was taken as 'time zero'. Eggs were placed at a constant 20 °C during development. These insects were sampled at specific timepoints during the pupal stage. These timepoints are measured in accumulated degree hours (ADH).

### *2.2. RNA extraction, cDNA preparation and location of potential markers*

Total RNA was extracted from pupal samples using TRIzol reagent (Invitrogen) according to manufacturer's instructions. A sample from the end of the larval stage was also included for comparison. The extracted RNA was reverse transcribed to cDNA (Omniscript reverse transcriptase kit, Qiagen). Potential markers were located by the use of differential display—the random amplification of cDNA (GeneFishing™ DEG 100 Kit, SeeGene). Fragments were visualised on an agarose gel and differences in banding pattern were extracted, sequenced using an ABI 310 Genetic Analyser (Applied Biosystems) and primers designed from the sequences. The differentially expressed gene discovered in this work, nominated Gene G, was quantified, along with two other potential differentially expressed genes (Arylphorin receptor and LSP-2 gene) sequenced in previous research [2,3]. Actin was also included for normalisation [4].

### *2.3. Quantification of gene expression with real time PCR*

The expression of the four genes was quantified in cDNA samples from various pupal timepoints using real-time PCR amplification. The primers for each QuantiTect® Custom Assay (QIAGEN) were G Gene CGCAAAGCCCAACAAGAA and TTGGCA CGGAATTTGCTGATG, Arylphorin receptor CAGACAATGCAGGGTATAAGAG and GGGGCATATAGACCTGATGTAA, LSP-2 GCGAAATTCCACACTTCAACA and GCGAGCACCATAGTATTCGAT, Actin TCAAGTCATCACCATCGGTAA and ACCGCAAGATTCCATACCCAA. Samples were amplified and quantified on the ABI Prism 7000 (Applied Biosystems).

## 3. Results

The CT values produced after real-time PCR amplification were normalised with actin values and then compared with the gene expression values at the end of the final larval stage. The values illustrated in Fig. 1 are therefore ratios of pupal timepoint gene expression against larval gene expression.

The Arylphorin receptor gene is highly expressed at the beginning of the pupal stage (peak expression is approximately 500 times that of the end of the larval phase). Gene G is expressed at level similar to that of the late larval sample until the end of the pupal stage when expression

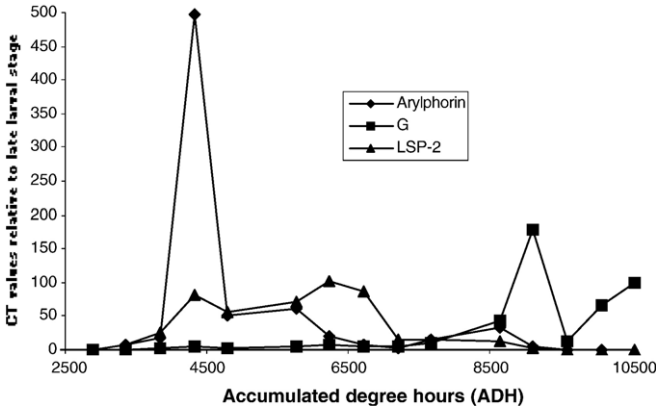


Fig. 1. Expression of Arylphorin receptor, Gene G and LSP-2 genes at various ADH timepoints throughout the *C. vicina* pupal stage compared to expression at the end of the larval period.

increases approximately 200-fold. LSP-2 is not as highly expressed as the other two genes. This gene is expressed through the middle of the pupal stage, but not at the beginning or end.

## 4. Conclusion

This preliminary research has indicated that the differing expression of genes in the developmental lifecycle of *C. vicina* can be used to age immature insects. Increased levels of Arylphorin receptor gene relative to the larval stage indicate pupae of 4500 ADH. Similarly, increased expression of Gene G indicates pupae older than approximately 8640 ADH. Increased LSP-2 expression does not provide a precise indication of pupal age. Further work is required to find more precise markers for the rest of the pupal stage and also for the complete *C. vicina* lifecycle.

## Acknowledgments

The authors would like to acknowledge the time, advice and equipment provided by Dr. Matthew Arno, Genome Centre, King's College London. Thanks also to Steve Tann-Ailward for manuscript advice.

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