Journal of Forensic Research
ISSN: 2157-7145

Abdel-Ghaffer et al., J Forensic Res 2014, 5:5
DOI: 10.4172/2157-7145.1000238

Research Article Open Access

Age-Dependent Gene Expression of Blow Fly *Lucilia cuprina* (Diptera: Calliphoridae) during Egg Development Improving Age Estimation in Forensic Entomology

Abdel-Ghaffer H[1], Mennatallah Abd-Elmottelb[1] and Amira Zaky[2]*

[1]*Department of Zoology, Faculty of Science, Alexandria University, Alexandria, Moharram Bake, P.O Box 21511, Egypt*
[2]*Department of Biochemistry, Faculty of Science, Alexandria University, Alexandria, Moharram Bake, P.O Box 21511, Egypt*

Abstract

Estimating the age of immature stages of necrophagous flies developing on corps, may provide clues for understanding the minimum post-mortem intervals (PMI) to aid death investigation. It can be hard to comprehend precisely the interval age of developmental stage that does not increase in size including egg. In this study we investigated the feasibility of predicting egg age from ovi-position to hatching over 2 hour's intervals of egg development. The expression profile of three genes, cuticle I, Ecdysone receptor (EcR) and chymotrypsin of *lucilia cuprina* (Diptera: Calliphoridae) were determined at 2, 6, and 8 hours of egg development. Semi-quantitative reverse transcriptase (RT)-PCR were applied for profiling of indicated genes expressions from eggs of different developmental stages by designing specific primers sets. Target transcripts were then quantified from the gel using image J and syn gene programs. The results indicated time-dependent expressions of the three genes with significant increase at the selected time points. Cuticle 1 expression was increased up to 2, 25 and 6 folds at 4, 6 and 8 hours respectively when compared to 2 hours expression level. Likewise a significant induction of both chymotrypsin and EcR (around 1.2, 1.7 then 1.2 and 3, 2, 6 folds, respectively) during 4, 6 and 8 hours were observed. Collectively our results indicate that developmental eggs exhibit differential and specific time-dependent expression profile of target genes that can contribute as measurable factors in future investigations.

Keywords: *Lucilia cuprina (L. cuprina);* Reverse transcriptase (RT)-PCR; Ecdysone receptor (EcR)

Introduction

Entomological evidence has long been used to aid investigators in the estimation of a postmortem interval (PMI) [1,2]. Such estimates are possible for necrophagous flies, especially blow flies (Diptera: Calliphoridae), as they are capable of colonizing remains soon after death and progress through a well-understood set of developmental stages, including the egg, three larval instars pupation and eclosion as an adult fly.

PMI estimates are based primarily on age calculation of the eldest immature developmental stages [3,4]. To date, age estimation methods are frequently focused on the larval stage with little published data for the age estimation of pupae, and egg, indicating the need of adding new tools that can aid in assessment of fly developmental stage data in order to generate more precise PMI [5].

Being the shortest developmental stages that do not increase in size, age estimation is an obstacle; hence other traits including the differential expression of genes may offer a source of data estimating blow fly age. In this study the alterations in gene expression profile throughout development, represent a potential useful data source for objectively identifying smaller size stages of egg development. Therefore we detected expression levels of specific genes: ecdysone receptor (EcR), cuticle I and chymotrypsin of *L. cuprina* egg in parallel to embryonic age at specific points in time at 30°C through selected hours.

Materials and Methods

Rearing of eggs

Adults of *L. cuprina* flies were held in an insectary at 25 ± 1°C with approximately 70-80% relative humidity (RH) and photo-period of 16:8 h light-dark cycle. Newly emerged flies (Ca-200) were caged, these flies were fed a 1: 1 mixture of dry granular sugar and powdered milk in Petri dish. Fresh water was supplied via jar with small pieces of plastic foam on the surface, were the colony was then maintained.

Sampling

The fly cage was presented with beef liver at room temperature (RT) and examined every 15 min. adult females were closely observed laying eggs, in order to study the duration of the egg stage at zero time (I.e time of oviposition). The egg masses were harvested and incubated at constant 30°C in Petri dishes (considering egg mass temperature). The eggs were checked at 30 min intervals and triplicates were collected for each 2 hours interval period to hatching. The collected samples were washed thoroughly with phosphate buffer saline (PBS) then maintained at -80°C prior to RNA extraction.

Isolation of total RNA

Total RNA was extracted from *L. cuprina* eggs, according to Chomczynski and Sacchi (6) using GStractTM RNA Isolation Kit II Guanidinium Thiocyanate method.

Egg masses were homogenized in (TRI) reagent (80-100 mg tissue/ml) and incubated at room temperature (RT) for 10 minutes. Two

***Corresponding author:** Amira Zaky, Department of Biochemistry, Faculty of Science, Alexandria University, Alexandria, Moharram Bake, P.O Box 21511, Egypt, Tel: (+20)1223584529; Fax: +203-3911794; E-mail: amzakyha@yahoo.com

Received May 12, 2014; **Accepted** August 27, 2014; **Published** August 30, 2014

Citation: Abdel-Ghaffer H, Abd-Elmottelb M, Zaky A (2014) Age-Dependent Gene Expression of Blow Fly *Lucilia cuprina* (Diptera: Calliphoridae) during Egg Development Improving Age Estimation in Forensic Entomology. J Forensic Res 5: 238 doi:10.4172/2157-7145.1000238

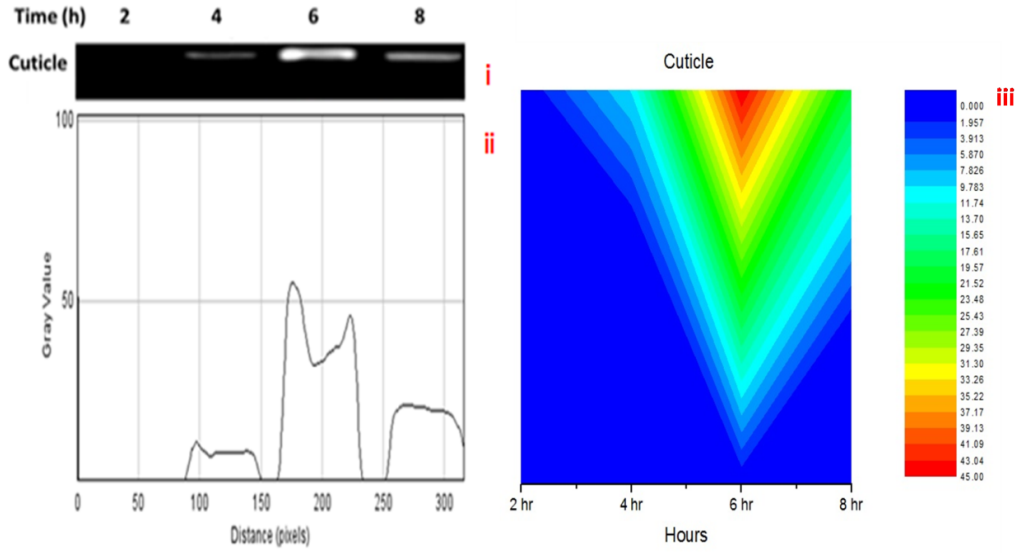

Figure 1: Cuticle **I** expression during egg development. (i) Shows RT-PCR results of cuticle **I** gene modulation that were quantified using image J program (ii) also corresponding heat map is presented (iii).

hundred microliters chloroform were added to the homogenate and mixed by vigorous vortexing for 20 s and incubated at RT for 5 min then centrifuged at 12,000 rpm for 15 min. The aqueous phase was transferred into new test tube and 0.5 ml isopropanol was added, mixed and incubated at RT for 10 min. The mixture was then centrifuged at 12,000 rpm for 20 min and the aqueous/isopropanol solution was aspirated. The pellets were washed twice with ice-cold 70% ethanol solution then was left to dry. The dried pellets were re-suspended in 50-100 µl RNAase free H_2O and stored at -80°C.

Reverse transcriptase PCR analysis

Alterations in the mRNA levels of genes relevant to different developmental stages were determined using semi-quantitative reverse-transcriptase PCR (RT-PCR) using one-step RT-PCR (RT/PCR Master Mix. Gold Beads, BIORON). Briefly, total RNA (1-3 µg) and random primer (3 µM) mixture were denatured at 70°C for 5 min and placed on ice. The incubated mixture was added to the RT/PCR Gold mix that contains all the components necessary for cDNA synthesis and amplification in one tube. The cDNA synthesis reaction was performed at 42°C for 60 min then 5 min at 94°C for RTase inactivation. Specific primers were used for amplification of target gene(s) (chymotrypsinogen F- CCGGTGATGAGGTTATTGCT: R- ACTCCAGAGGATGCCAACAC, EcR: F-TTTCACCCTCGAGCAGTCTT: R-CGTCGAAAGAACCCCTTACA, cuticle **I**: F-TCAAGCTGCTTCTGGTGATG: R-CCTTCAAGATGGCTTCAGGA) then subjected to PCR cycles, each cycle consisting of denaturation, annealing and extension. Annealing temperature and time were optimized for each primer/template combination. PCR producted were separated on 2% agarose gel then bands were analyzed using image J and Syn gene programs.

Results

Cuticle I embryonic expression in hours

The gene expression profile variations at 30°C through embryonic development of egg at zero time to hatching was analyzed by the RT-PCR, products on the gel illustrating different band intensities as shown in Figure 1 using the image J program (gray value), further the Syn Gene program was applied to interpret and confirm the results obtained qualitatively and quantitatively throughout egg embryogenesis. The results showed that the expression of embryonic cuticle I displayed a consistent pattern. The fluctuations of cuticle I expression in samples of 2, 4, 6 and 8 hours old were quantified and verified by the gel electrophoresis. The expression was affected and the band displayed an increase at 6 hour, were a declination was observed at 8 hours indicating that the embryonic cuticle I was highly deposited at 6 hours interval from zero time (Figure 1). Figure 1 demonstrates the cuticle I expression state plotted during development in hours, while the Syn Gene program (Figure 1) shows the moderately significant association with age-dependent expression through hours, as displayed by different intensities of bands referring to color, indicating the increase of expression at 6 hours (Figure 1 heat map). Quantitative analysis of the results was performed using image J software. Expression levels were calculated in reference to 2 h level in which we observed significant induction ($p<0.01$) of cuticle I gene around 2, 25 and 6 folds at 4, 6 and 8 hours (Figure 4).

Significant expression of changes of chymotrypsin associated to age of the egg

A useful way to estimate the embryonic development of egg age, is analyzing genome-wide gene expression. Implication of the age-dependent gene expression was examined by analyzing the chymotrypsin expression in egg through the selected hours. RT-PCR analysis showed a moderate expression throughout 2, 4 and 8 hour, while 6 hour provoked a relatively increased expression (Figure 2). Consistently, fold expression of chymotrypsin over 4, 6 and 8 hours relative to 2h levels, showed around 1.2, 1.7 folds increase, respectively (Figure 4).

Indicative time period of EcR expression during egg development

Further study was carried out on the EcR expression, to understand the age-related gene expression changes in *Lucilia cuprina* egg. To estimate the age through the level of the EcR expression, the experimental set up of RT-PCR was held from zero time of egg laying

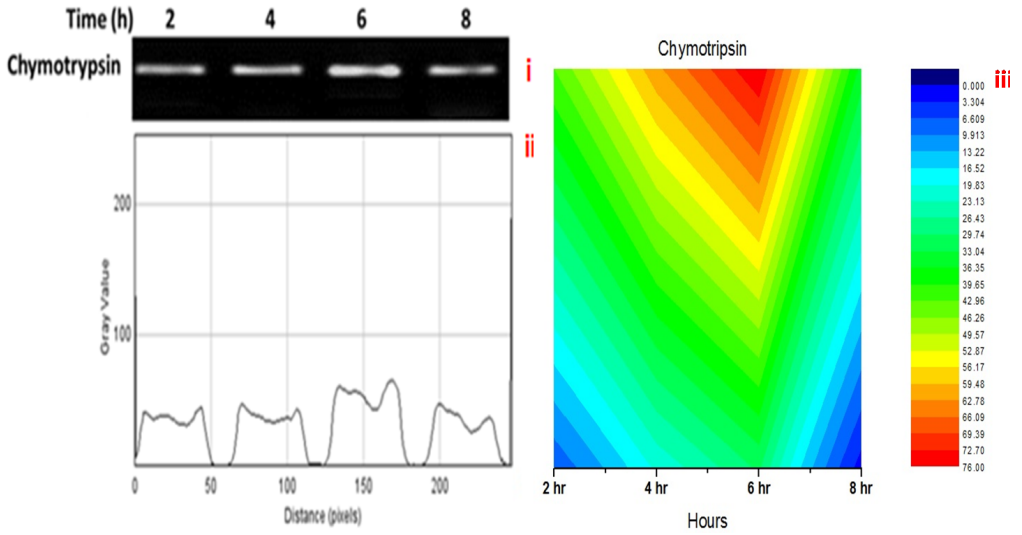

Figure 2: Chymotrypsin protein level modulation throughout egg development. (i) Indicate chymotrypsin gene expression level measured by RT-PCR, (ii) shows the bands quantification using image J program and (iii) is the corresponding heat map.

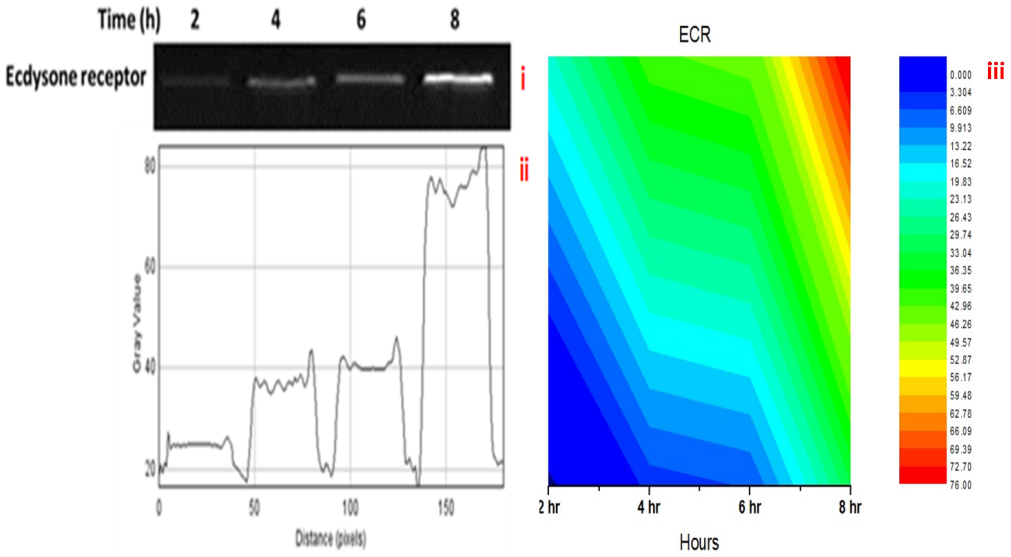

Figure 3: Ecdysone receptor gene expression profile in developing *L. cuprina* egg. (i) Shows time-dependent induction of ecdysone receptor gene over 8 hours of egg development. RT-PCR bands were further analyzed quantitatively using image J program (ii) also corresponding heat map (iii) is shown.

to hatching at an interval of 2, 4 , 6 and 8 hours. At this condition EcR profile displayed maximum expression at 8 hours (Figure 3) compared to other experimented hours, which was also well demonstrated in the array and gel electrophoresis (Figure 3). Moreover quantitative estimation of EcR gene expression showed around 3, 2 and 6 folds increase ($p<0.01$) at 4, 6 and 8 hours respectively when calculated from 2 h expression value (Figure 4).

Discussion

Eggs were chosen because there was no quantitative means of measuring their degree of maturity and if aging is attempted at all investigation of embryos, to relatively divide the stage [2].

In the current report we selected cuticle I, Ecdyson receptor (EcR) and chymotrypsin genes to be investigated as molecular markers during the first 8 hours of egg maturation.

It has been shown that control of cuticle differentiation and production relies connectively on transcriptional regulation [6,7]. The properties of cuticle vary greatly during egg development according to the assembly of chitin and cuticle proteins of the insects' exoskeleton. Detecting the expression of cuticle I gene was an attractive approach to estimate the age of embryonic egg per hours. The results revealed a peak induction at 6 hours, indicating that the embryonic cuticle I was highly deposited at this period. Similar results obtained by Tarone and Foran [1] in which they showed that egg masses <2 h old did not express chitin synthase, while egg masses older than 6 h were expressed.

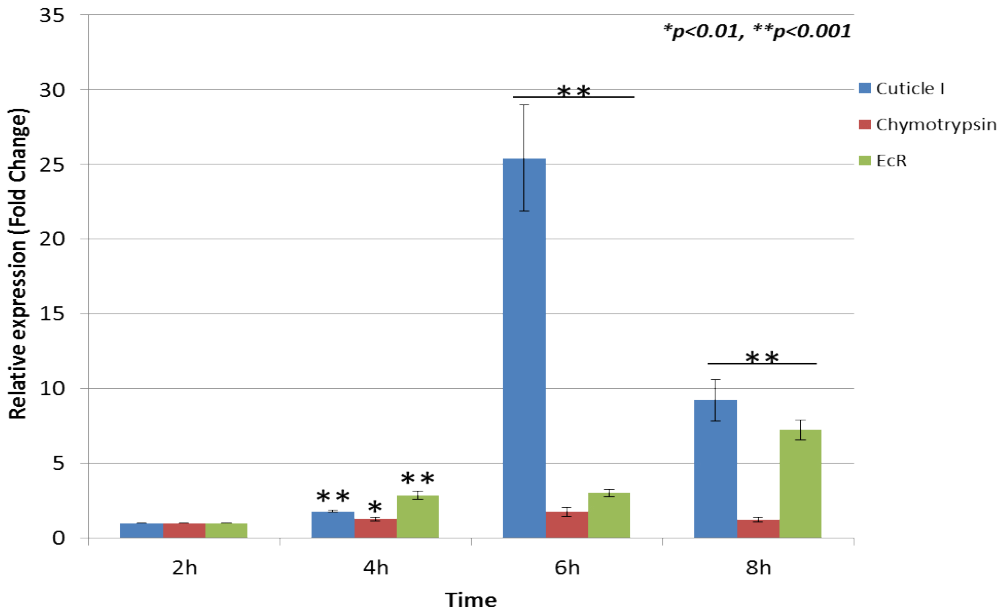

Figure 4: Relative expressions of Cuticl I, Chymotrypsin and Ecdysone receptor genes calculated as fold change from 2 h results.

Bale et al. [8] mentioned that disruption of gene expression showed poor cuticle integrity when embryos are mechanically devitalize, which is in agreement with our results as a reduction was observed at 8 hours developing on protein synthesis, suggesting that the cuticle deposition before hatching will decrease. Other physiological studies demonstrated that this reduction was required for the degradation of the normal progression during moulting cycles [9,10].

The investigation was extended to analyze the expression of chymotrypsin that found to increase throughout development in a time-dependent manner with maximum induction at 6 hours. In condition the interaction between temperature and age proved to have a significant impact on gene expression, which is in agreement with Boheme et al. group [11], thus reflects the dependency of gene expression on accumulated degree hours related to egg development as the amount of hours accumulated at a certain age correlates to the level of completion of development.

Furthermore primers for EcR, one of age-related genes, were also designed for the proper assessment of embryonic egg development. Ecdysteroids are sterol hormones that play an important role in arthropod development, growth, and reproductive processes, indicating the control of *previtellogenic* and vitellogenesis processes. It was reported that they regulates molting by activating the heterodimer formed by the ecdysone receptor [12], as well as ecdysone signaling that controls the morphogenetic movements of the embryo. The effect of ecdysteriods is mediated by its binding to ecdysone receptor EcR [11]. The way that are limited to invertebrates that molt makes they well suited to regulate the expression of transgenes [11,12]. Reverse transcriptase PCR (RT-PCR) was performed here to test the EcR expression in egg mass at selected time as the embryonic development of *Lucilia cuprina* last for about 9 hours. The results indicted maximum expression of EcR at 8h which is portably correlated to the need for high protein level and function during embryogenesis. Previous reports showed that EcR complex bind to ecdysone response elements of DNA and acts as transcription factor, and it has been demonstrated that rising of ecdysteroid level in insect`s titer in haemolymph can induce its own receptor expression, which in turn mediates transcriptional events [13].

The blow fly *lucilia cuprina* was chosen herein for its global distribution and forensically significant value in estimating post mortem intervals [2,11]. The general principle in the current study was designed to address and investigate aging of one of the shorter developmental stages, the egg, via gene expression as quantitative means for measuring their degree of maturity to help calculating the PMI. In conclusion this report highlights the differential pattern of genes expressions in growing egg as novel forensic tools in aging detection methods that still needs further investigation.

Acknowledgment

This work is supported partially by BA/CSSP-2010 grant. Authors would like to acknowledge the support of Alexandria University.

References

1. Tarone AM, Foran DR (2011) Gene expression during blow fly development: improving the precision of age estimates in forensic entomology. J Forensic Sci 56 Suppl 1: S112-122.
2. Tarone AM, Jennings KC, Foran DR (2007) Aging blow fly eggs using gene expression: a feasibility study. J Forensic Sci 52: 1350-1354.
3. Davies K, Harvey ML (2013) Internal morphological analysis for age estimation of blow fly pupae (Diptera: Calliphoridae) in postmortem interval estimation. J Forensic Sci 58: 79-84.
4. Amendt J, Richards CS, Campobasso CP, Zehner R, Hall MJ (2011) Forensic entomology: applications and limitations. Forensic Sci Med Pathol 7: 379-392.
5. Zehner R, Amend J, Boehme P (2009) Gene expression analyses as a tool for age estimation of blow fly pupae. Forensic Science International: genetics supplement series 2: 292-293.
6. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-159.
7. Moussian B (2010) Recent advances in understanding mechanisms of insect cuticle differentiation. Insect Biochem Mol Biol 40: 363-375.
8. Bale JS, Masters ID, Hodkinson C, Awmack TM, Bezemer VK, et al. (2002) Herbivory in global climate change research: direct effects of rising temperatures on insect herbivores. Global Change Biology 8:1-16.

9. Charles JP (2010) The regulation of expression of insect cuticle protein genes. Insect Biochem Mol Biol 40: 205-213.

10. Greenberg B, Kunich JC (2002) Entemology and the law I flies as forensic indicators, Cambridge.

11. Boheme P, Spahn P, Amendt J, Zehner R (2012) Differential gene expression during metamorphosis: a promising approach for age estimation of forensically important calliphora vicina pupae (Diptera: Calliphoridae). Int J legal Med 127: 243-249.

12. Tarrant AM, Behrendt L, Stegeman JJ, Verslycke T (2011) Ecdysteroid receptor from the American lobster Homarus americanus: EcR/RXR isoform cloning and ligand-binding properties. Gen Comp Endocrinol 173: 346-355.

13. Sun X, Song Q, Barrett B (2003) Effects of ecdysone agonists on the expression of EcR, USP and other specific proteins in the ovaries of the codling moth (Cydia pomonella L.). Insect Biochem Mol Biol 33: 829-840.