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

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**Abstract**

Estimating the age of immature stages of necrophagous flies developing on corpses, 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 oviposition 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, 2.5 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.

**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
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, whereas 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, 2.5 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).

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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 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).
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, Ecdysone 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.
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 regulate 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 ecdysteroids 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.

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