Expression of HO and ALAS mRNA in blue- and white-shelled ducks (1)

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Abstract

The native Brown Tsaiya duck (*Anas platyrhynchos*) is a major laying duck breed in Taiwan, and their eggshell color is varied from white to dark blue. It is possible that the shell gland of blue-shelled ducks (BSD) can accumulate higher concentration of biliverdin than white-shelled ducks (WSD) do. However, there is no strong evidence to prove this assumption. The mRNA expression level of heme oxygenase (HO) and delta-aminolevulinate synthase (ALAS1) of shell gland of BSD and WSD was analyzed in this study. Reverse transcriptase-polymerase chain reaction was performed with total RNA extracted from epithelium cells of shell glands of Tsaiya ducks. The partial cDNA clones of HO1, HO2 and ALAS1 were 258 bp, 263 bp and 111 bp, respectively, and showed an average 91% similarity to chicken sequences. Ultraviolet spectrophotometery and HPLC were used to determine the biliverdin concentration in the serum and uterus liquid at 6, 12, 15, 18, 20, 22 and 23.5 h post oviposition. Real time PCR was applied to analyze these gene expression of liver and shell gland in BSD and WSD. The expression of HO and ALAS1 mRNA of shell glands in BSD and WSD during the ovulation period did not have significantl difference, with exception at 12 h of ALAS1, HO2 in shell gland and HO2 in liver. Results of this study also showed significant differences in the biliverdin concentration in the uterine fluid. Such variations may arise from inconcurrent of mRNA concentration and encoded gene product (enzyme) activity or existence of a mechanism that controls the biliverdin concentration difference of uterine fluid between BSD and WSD.

Key Words: Blue-shelled duck, Biliverdin, Heme oxygenase, Aminolevulinate synthase.

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INTRODUCTION

The pigmentation of avian eggshell is a complex process controlling by two types of pigments, biliverdin and protoporphyrines. Biliverdin, a green pigment, is a byproduct of hemoglobin breakdown and will eventually exhibit a blue and/or green color on the eggshell. In contrast to biliverdin, protoporphyrines is an immediate precursor of the heme molecule, and will give eggshells a reddish and/or brown color. The native Brown Tsaiya duck (*Anas platyrhynchos*) is a major laying duck breed in Taiwan. It is one of the highest laying duck breeds in the world (Tai *et al.*, 1989; Chen *et al.*, 2003). The eggshell colors of Brown Tsaiya vary from white to dark blue. White color is the major eggshell color of White Tsaiya duck. Blue eggshells contain mainly biliverdin and a small amount of protoporphyrin. White eggshells contain only protoporphyrin (Liu *et al.*, 1998). The genes involved in color polymorphism must exert their influence via enzyme systems that produce different pigments in the eggshell. There are three enzymes of the major importance in relation to egg shell pigment formation (Schwartz *et al.*, 1975 as cited in Shoffner, 1981). The primary determinant of protophyrin pigment synthesis is delta-aminolevulinate synthase (ALAS1), the initial rate-limiting enzyme in the pathway. The uteri of brown eggshell females have three to five times more ALAS1 activity than the uteri of the translocation mutant females. The translocation females, however, have about twice as much ferrochelatase uterine activity and nearly four times as much heparic activity as those females with standard chromosomes one and Z, laying brown shelled eggs. Heme oxygenation is the rate-limiting step for heme degradation, which produces biliverdin (Schwartz *et al.*, 1980). It is possible that the shell gland of blue-shelled ducks (BSD) can accumulate much higher concentration of biliverdin than that of white-shelled ducks (WSD). It is our aim to know whether the higher mRNA levels of heme oxygenase (HO) and ALAS1 found in the BSD shell gland is responsible for the higher concentration of biliverdin.

MATERIALS AND METHODS

I. Animals and sample collection

A total of 42 ducks including 21 laying Brown Tsaiya ducks with blue-shelled eggs and 21 White Tsaiya ducks with white-shelled eggs were used in this study. Ducks were caged individually and fed *ad libitum* on a commercial layer diet (CP 19%, ME 2700 kcal/kg). They were subjected to an artificial photoperiod of 14 h light: 10 h darkness. When ducks reached 72 wks of age, the oviposition was monitored every 10 min during the night preceding sampling. Three Brown Tsaiya ducks and three White Tsaiya ducks were selected randomly and sacrificed by electrical shock at 6, 12, 15, 18, 20, 22 and 23.5 h post oviposition. The shell gland was carefully disected to collect the uterine fluid for biliverdin concentration analysis. The tissue samples, shell gland and liver, were frozen in liquid nitrogen and stored at -80℃ until RNA extraction.

II. Isolation of total RNA from liver and shell gland

The shell gland and liver of Brown Tsaiya duck were ground with liquid nitrogen into powder and then weighted. Tissues, 50 mg each, were homogenized with 1 mL RNA-Bee^{TM} RNA Isolation Reagent (Tel-Test, Inc, Friendswood, Tx) and incubated at RT for 10 min. Total RNA was extracted according to

the manufacturer's instructions. The preliminary quantity and purity of the extracted total RNA were determined by using a spectrophotometery (Unicam, U.K.).

III. Cloning of duck specific heme oxygenase and delta-aminolevulinate synthase genes

The consensus sequences of each target genes, HO1, HO2, ALAS1, were extracted from alignments of human, mouse and chicken gene sequences in Genbank using SeqWeb Version 2.1 (Wisconsin Package). Three pairs of degenerate primers were designed to amplify HO1, HO2 and ALAS1 cDNA fragment. Cycling parameters for PCR amplification were one cycle of 50℃ for 30 s and 94℃ for 2 min followed by 35 thermal cycles of 94℃ for 1 min, 45℃ for 45 s and 72℃ for 45 s and a final extension step at 72℃ for 5 min. The amplified products were fractionated on a 2% agarose gel and visualized by ethidium bromide staining. PCR products were then sliced out, purified (QIAquick Gel Extraction Kit, Qiagen GmbH, Hilden, Germany), cloned (pGEM-T easy vector system, Promega, USA) and sequenced (Seeing Bioscience Co. Ltd., Taipei, Taiwan. http://www.seeingbioscience.com).

IV. Reverse transcription real-time polymerase chain reaction

Real time PCR was performed by using a set of Power SYBR® Green PCR Master Mix Kit (Applied Biosystems, USA) and a Real-Time PCR System (ABI 7000, Applied Biosystems, USA). Reactions were carried out according to the instruction manual but were scaled down to 25 μL per reaction. Each reaction, containing 50 ng of template, was performed in duplicates, and β-actin mRNA was used as the internal control. The thermal cycling parameters for amplification were slightly modified: 50℃ for 2 min and 95℃ for 10 min, then 95℃ for 15 s, 60℃ for 1 min for 40 thermal cycles, with a final dissociation stage, 95℃ for 15 s, 60℃ for 30 s and 95℃ for 15 s, to complete the melting curve acquisition. The mRNA levels were quantified using interpolation method according to the standard curve constructed with serially diluted standard templates. The data were normalized with β-actin to account for the differences in reverse transcription efficiencies and the amount of template in the reaction mixtures.

V. Analysis of biliverdin concentrations

The collected serum and uterine liquid were dried by using a centrifugal vacuum pump and then resuspended with diluent (50% 3 N HCl and 50% acetonitrile, HPLC grade, J. T. Baker, USA). All samples were centrifuged at 18,350 x g for 10 min. The supernatants were analyzed by HPLC equipped with an L-4500 diode array detector, L-6200A intelligent pump, D-6000 interface (Hitachi, Japan) and Hamilton PRP-1 reversed phase (10 μm particle size, 4.1 mm × 250 mm) column. The flow rate was set as 2.0 mL/min and a solvent gradient elution was applied. The initial mobile phase composition was 20% acetonitrile and 80% 0.1 M ammonium acetate (pH 5.2) (Sigma, Germany) for 2 min. The solvent gradient consisted in a 16 min linearly change to 100% acetonitrile, followed by 2 min at 100% acetonitrile. The total runtime was 20 min. Standards were prepared as samples. Concentrations in the 6 calibration points were 31.25, 62.5, 125, 250, 500, and 2000 nM with R values of 0.9995.

VI. Statistical analysis

All values are given as mean ± SEM. Values were first analyzed with one-way ANOVA, and the

comparison of means among groups was performed using Tukey's honest significant difference test. All of these analyses were performed with the SAS 9.1.3 package (SAS Institute,1999).

RESULTS

Partial cDNA clones were obtained and confirmed for HO1, HO2 and ALAS1 in Brown Tsaiya and showed an average of 91% similarity to chicken sequences (Table 1). The BSD and WSD biliverdin concentrations at 6, 12, 15, 18, 20, 22, and 23.5 h post oviposition in uterine fluid were shown in Fig. 1. The biliverdin concentration in the BSD uterine fluid declined after 6 h and reached the lowest level at 15 h, then increased linearly to 23.5 h post oviposition. The same pattern was observed in the WSD. The BSD showed significantly higher biliverdin concentration in the uterine fluid than the WSD ($p<0.05$), except at 12 and 23.5 h.

There is no significant difference of serum biliverdin concentraction found between the WSD and BSD. However, the serum biliverdin concentration of WSD tended to be higher than that of BSD at 12, 20, and 22 h postoviposition, while to be lower at 6, 18, and 23.5 h postoviposition (Fig. 2).

Lower ALAS1 mRNA expression in the shell gland was observed in BSD when compared with the WSD (Fig. 3a). The ALAS1 mRNA levels decreased from 12 to 15 h postoviposition, and increased from 15 to 22 h postoviposition then decreased again to 23.5 h in WSD. However, this pattern was not observed in the BSD. Instead, its ALAS1 mRNA levels decreased from 6 to 12 h, then increased to 20 h and maintained till 23.5 h postoviposition. The levels of ALAS1 in liver at various time points postoviposition of BSD and WSD were showed in Fig. 4a. There is no significant difference found both in the shell gland and liver ALAS1 mRNA levels between these two breeds.

In liver, the levels of HO1 mRNA at various time postoviposition in the BSD were higher than that of the WSD, with exceptions at 6 and 18 h postoviposition. Also, similar tendency of HO1 mRNA expression pattern in shell gland was observed between BSD and WSD (Fig. 3b and 4b). It gradually decreased from 6 h and reached the lowest point earlier at 12 h and 15 h in WSD and BSD, respectively, then increased to 23.5 h postoviposition. However, no significant difference of HO1 mRNA in liver and shell gland were observed between BSD and WSD.

In the shell gland, the HO2 mRNA expression levels in WSD tended to be but not significantly higher than these of the BSD. However, the expression of HO2 mRNA in the shell gland of BSD was significantly higher that of the WSD at 12 h postoviposition (Fig. 3c). Similar expression pattern of HO2 mRNA was also found in the liver (Fig. 4c).

Table 1. Sequence similarity analysis of duck HO1, HO2 and ALAS1

Anas platyrynchos	*Gallus gallus* (Accession No.)	*Homo sapiens* (Accession No.)	*Mus musculus* (Accession No.)
HO1	91 (NM_205344)	70 (NM_002133)	70 (NM_010442)
HO2	92 (NC_000911)	76 (NM_001127206)	77 (NM_010443)
ALAS1	91 (NM_001018012)	80 (NM_000688)	75 (NM_020559)

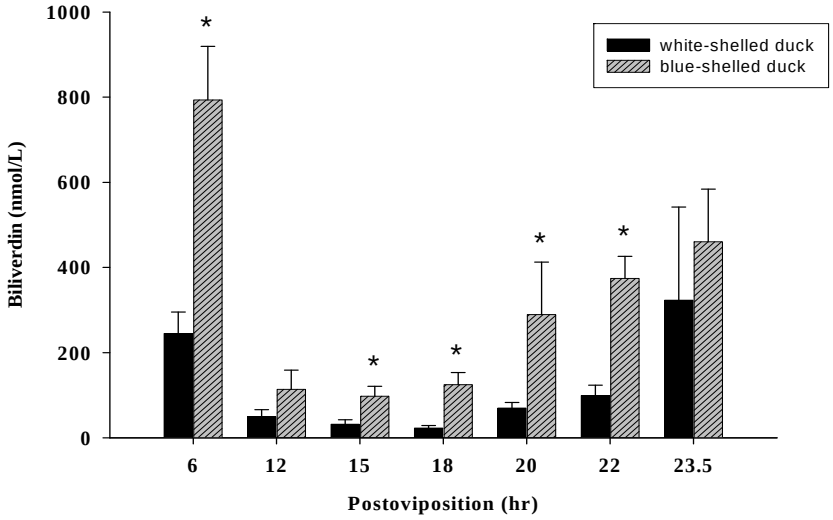

Fig. 1. The concentration of biliverdin in uterine fluid at various time points postoviposition for BSD and WSD.
*Significantly different between blue-shelled and white-shelled ducks at the same hour postoviposition, $P < 0.05$.

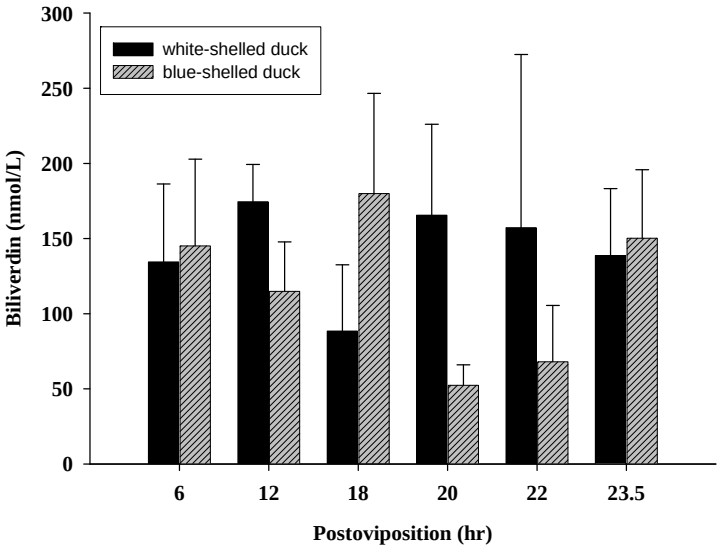

Fig. 2. The concentration of biliverdin in serum at various time points postoviposition for BSD and WSD.

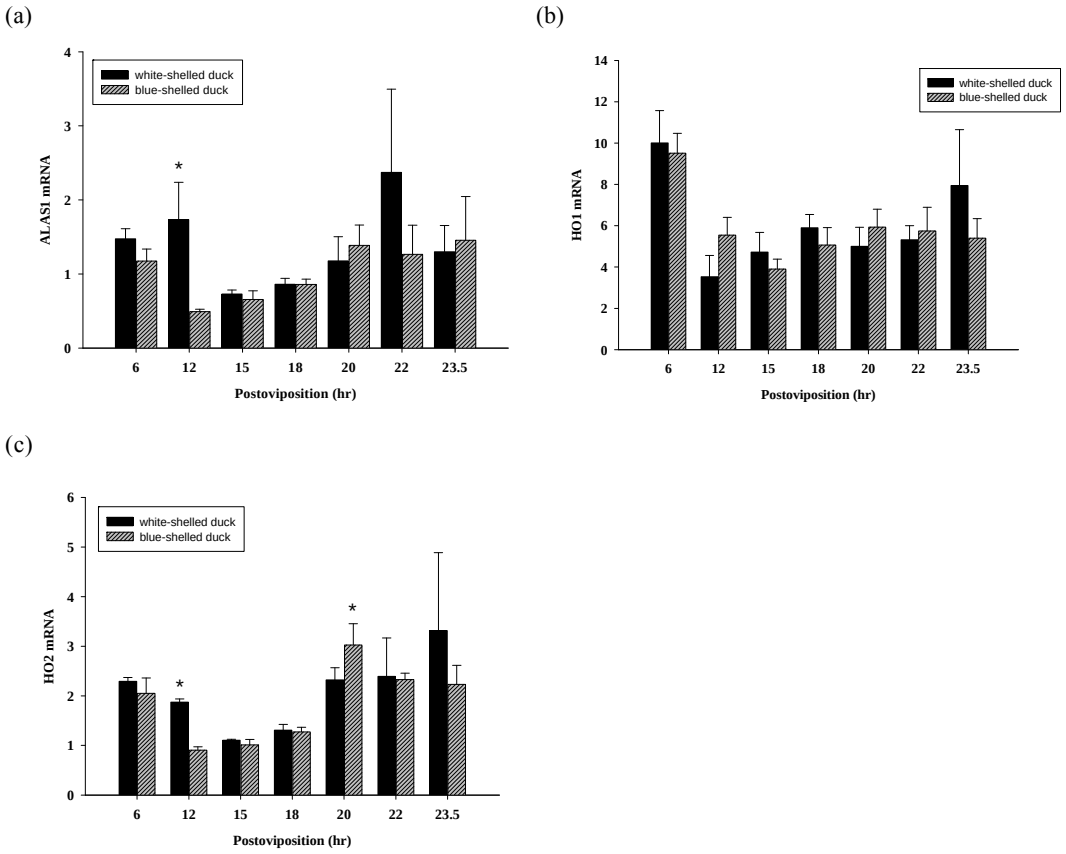

Fig. 3. The concentration of ALAS1(a), HO1(b) and HO2 (c) mRNA in shell gland at various time points postoviposition for BSD and WSD.

*Significantly different between blue-shelled and white-shelled ducks at the same hour postoviposition, $P < 0.05$.

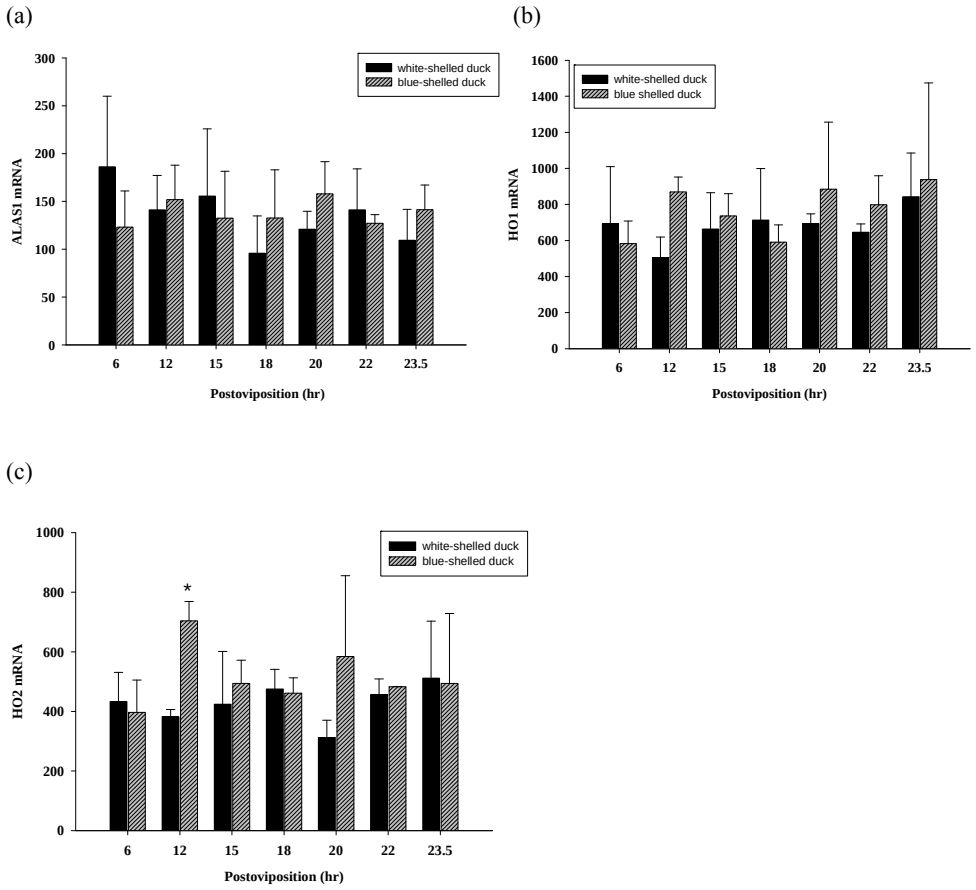

Fig. 4. The concentration of ALAS1(a), HO1(b) and HO2 (c) mRNA in liver at various time points postoviposition for BSD and WSD.

*Significantly different between blue-shelled and white-shelled ducks at the same hour postoviposition ($P < 0.05$).

DISCUSSION

In the present study, significant differences of the biliverdin concentration were found in the uterine fluid but not in the serum between BSD and WSD. Previous studies showed either blood (Kennedy and Vevers, 1973; Lang and Wells, 1987) or shell gland could be the origin of shell biliverdin, but still no direct evidence to support either of the hypotheses. Zhao *et al.* (2006) demonstrated birds that lay blue shelled eggs may have the ability to synthesize biliverdin in the shell gland. They reported a significant difference in shell gland biliverdin concentration between blue shelled (8.25 ± 2.55 nmol/g) and brown shelled (1.29 ± 0.12 nmol/g) chickens. If this were true, then blood may not be the only site of biliverdin synthesis for egg shell pigmentation. Our result was partially in agreed with the work of Zhao *et al*. (2006).

The concentration of ALAS1 mRNA was higher in liver than the shell gland of both BSD and WSD. While Polin (1957) reported that uterine tissues produce twice as much porphrin from delta-aminolevulenic acid compared to liver tissue *in vitro*, and the ALAS1 activity of brown-shelled chicken was higher in uterus than that of liver does (Schwartz *et al.*, 1980). The uteri of brown egg shell females have three to five times higher ALAS1 activity than the uteri of the translocation mutant females (Shoffner, 1981). Schwartz *et al*, (1980) also determined that brown shelled hens have an excess of 100 times more protoporphyrin in the egg shells and White Leghorn about four times than that of the translocation mutant females. ALAS1 is rate limiting enzyme for biosynthesis of protoporphrin which was the major pigment of brown egg shells. The results showed that no significant difference in ALAS1 mRNA between these two breeds during the ovulation period. Hens laying white eggs produce normal amounts of protoporphyrin in their shell gland but deposit only small amounts of this pigment in the shell (Etches, 1996). Hence, the ALAS1 mRNA expression level was not responsible for the different biliverin concentration in uterine fluid between BSD and WSD.

Heme oxygenase (HO) is the first and the rate-limiting enzyme of the microsomal heme degradation process which mostly occurs in the spleen, liver, bone marrow and kidneys; it yields biliverdin, carbon monoxide (CO), and iron as the final products (Kikuchi *et al.*, 2005). The static mRNA level of HO1 and HO2 in the shell gland was also been examined in our study, however, there was no significant difference observed. This result is consisted with what Zhao *et al.* (2007) reported on the genes of related enzymes in the porphyrin pathway applied in the BLAST search; none of these genes were found located inside the blue egg (*O*) locus. HO1 is an inducible isoform in response to stress such as oxidative stress, hypoxia, heavy metals, cytokines, *etc*. HO2 is a constitutive isoform which is expressed under homeostatic conditions. Both HO1 and HO2 are ubiquitously expressed and catalytically active. Higher level of HO1 mRNA, comparing to HO2, expressed in liver and shell gland of BSD and WSD, even without significant difference. The blue eggshell of duck contains mainly biliverdin and a small amount of protoporphyrin, whereas white eggshell contain only protoporphyrin (Liu *et al.*, 1998). Blue eggs are produced by hens that lack the ability to deposit significant amounts of protoporphyrin but are distinguished by their ability to deposit biliverdin (Etches, 1996). It is possible that ducks laying white eggs produce normal amount of biliverdin in their shell gland but deposit only small amounts of this pigment in the shell comparing with BSD.

In summary, no significant differences were observed in the level of HO1, HO2, ALAS1 mRNA expression and serum biliverdin concentration between BSD and WSD, only biliverdin concentration of uterin fluid from BSD shown significantly higher than WSD. These results may indicate mRNA levels are not equivalent to enzyme activity or there are other controlling mechanism responsible for the different biliverdin concentration of uterine fluid between BSD and WSD.

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生產青殼蛋與(或)白殼蛋鴨隻血基質氧化酶及胺基酮戊酸合成酶 mRNA 表現量(1)

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摘要

褐色菜鴨為台灣本土主要之產蛋品系，其蛋殼顏色可由白色、淡青色至深青色不等。鴨隻之產出青殼蛋者，其殼腺可能較產出白殼蛋者，累積有更高量之膽綠質，然釐清此等假設之有力試驗證據，迄今尚且付之闕如。本文旨在嘗試藉由殼腺中相關 mRNA 表現量之論證，以釐清鴨隻殼腺中具有較高活性之血基質氧化酶，對於青殼蛋之形成是否扮演關鍵性角色？應用反轉錄酶-聚合酶鏈鎖反應增殖菜鴨殼線上皮細胞中總 RNA，分別得到部分第一型、第二型血基質氧化酶及胺基酮戊酸合成酶 cDNA，長度分別為 258、263 及 111 bp，與雞者具 91%相似度。應用紫外光分光光度計與高壓液相層析儀偵測產青、白殼蛋鴨產蛋後 6、12、15、18、20、22 及 23.5 小時之子宮液及血清中膽綠質的濃度。結果顯示，就子宮液中含有之膽綠質濃度而言，鴨隻品系間確實呈現有顯著性之差異($P < 0.05$)。相對地，使用即時定量聚合酶鏈鎖反應就殼腺及肝臟中胺基酮戊酸合成酶、第一型及第二型血基質氧化酶 mRNA 之表現量，在產出青殼與白殼蛋之鴨隻間，彼此亦無顯著性之差異。至於造成產出青殼與白殼蛋之鴨隻間子宮液中膽綠質濃度差異，可能係因 mRNA 表現量多寡與其嗦定基因產物活性高低間並無直接關聯，抑或存有另一個調控機制所使然。

關鍵詞: 生產青殼蛋鴨隻、膽綠質、血基質氧化酶、胺基酮戊酸合成酶

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