



## Comparison of *HMOX1* expression and enzyme activity in blue-shelled chickens and brown-shelled chickens

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### Abstract

Blue egg coloring is attributed to biliverdin derived from the oxidative degradation of heme through catalysis by heme oxygenase (HO). The pigment is secreted into the eggshell by the shell gland. There is uncertainty as to whether the pigment is synthesized in the shell gland or in other tissues. To investigate the site of pigment biosynthesis, the expression of *heme oxygenase (decycling) 1 (HMOX1)*, a gene encoding HO, and HO activity in liver and spleen were compared between blue-shelled chickens ( $n = 12$ ) and brown-shelled chickens ( $n = 12$ ). There were no significant differences in *HMOX1* expression and HO activity in these tissues between the two groups. Since the liver and spleen, two important sites outside the shell gland where heme is degraded into biliverdin, CO and  $Fe^{2+}$ , did not differ in HO expression and activity we conclude that the pigment is most likely synthesized in the shell gland.

**Keywords:** biliverdin, blue egg, chicken, *HMOX1*.

Received: September 27, 2012; Accepted: March 13, 2013.

### Introduction

The blue color of some eggs is attributed to biliverdin (Kennedy and Vevers, 1973), a green tetrapyrrolic bile pigment that is secreted into the eggshell by the shell gland (Lang and Wells, 1987). Although the identity of blue egg pigment is well-known, the site of eggshell biliverdin synthesis has been disputed for many years. Some researchers believe that eggshell pigments are synthesized in the shell gland and cite as evidence for this the fact that (1) the shell glands of blue-shelled chickens contain higher levels of biliverdin than those of brown-shelled chickens (Zhao *et al.*, 2006; Wang *et al.*, 2009), (2) the activities of enzymes involved in porphyrin synthesis are higher in the shell gland of Rhode Island Red chickens that lay brown eggs than in a mutant strain laying white eggs that contain a negligible amount of porphyrin (Schwartz *et al.*, 1980) and (3) porphyrin pigments are present in epithelial cells of the shell gland during formation of the eggshell and fluctuations in their levels accompany eggshell formation (Tamura *et al.*, 1965). However, others have considered it improbable that the shell gland synthesizes such large amounts of porphyrin; in this case, blood is the most likely source of the pigment because of its ability to synthesize porphyrin that then reaches the shell gland via the circulation (Baird *et al.*, 1975).

Bile biliverdin is derived from the oxidative degradation of heme (Maines, 1997). In mammals, biliverdin is rapidly reduced to bilirubin by biliverdin reductase, secreted into the bile and ultimately excreted via the intestines. However, in birds, the low level of biliverdin reductase activity means that biliverdin is the main metabolite in bile pigment (Himes and Cornelius, 1975). In enterohepatic circulation, large amounts of bilirubin are absorbed into blood from the ileum and transported to the liver via the portal vein; little escapes the liver to reach the systemic circulation (Small *et al.*, 1972). However, the production of bilirubin that exceeds the livers capacity results in hemolytic jaundice which can reach other tissues via the blood to cause jaundice (Gilmore and Garvey, 2007). Similarly, excess biliverdin produced in blue-shelled chickens can reach the shell gland, leading to blue-colored eggs.

HO is a limiting-rate enzyme that catalyzes the degradation of heme to biliverdin, CO and  $Fe^{2+}$  (Maines, 1997). In view of the role of HO in the formation of biliverdin, *heme oxygenase (decycling) 1 (HMOX1)*, the gene encoding HO, is a key candidate gene for studying the molecular mechanism of blue egg formation. Previous studies of *HMOX1* expression in shell gland suggested that the blue egg coloring was associated with high gene expression in the shell gland of blue-shelled chickens (Wang *et al.*, 2010, 2011). However, the pattern of *HMOX1* expression in other tissues remains unclear. Hepatic and splenic reticuloendothelial cells are two important sites where heme is de-

graded to biliverdin (Hudson and Smith, 1975). Hence, analysis of *HMOX1* expression and HO activity in these tissues will be helpful in understanding the origin of eggshell biliverdin.

## Material and Methods

### Animals

Twelve Dongxiang blue-shelled chickens and 12 Dongxiang brown-shelled chickens were slaughtered 3–5 h prior to laying. Livers and spleens were collected and immediately stored in liquid nitrogen for further analysis. All chickens were provided by a local breed protection farm in Dongxiang town, Jiangxi province, China.

### RNA isolation, reverse transcription and real-time PCR

Total RNA was extracted with TRIzol reagent (TianGen Corp., Beijing, China). Two micrograms of total RNA was reverse transcribed into cDNA in a volume of 25  $\mu$ L consisting of 0.5  $\mu$ g of oligo(dT)15 primer, 5  $\mu$ L of 5xfirst-strand buffer, 5  $\mu$ L of 10 mM dNTP, 25 U of ribonuclease inhibitor, 200 U of M-MLV reverse transcriptase (Promega, Fitchburg, WI, USA) and RNase-free water to final volume of 25  $\mu$ L.

Real-time PCR was done in an ABI PRISM 7900 instrument (Applied Biosystems Corp., Foster, CA, USA). cDNA (1  $\mu$ L) was amplified in 20  $\mu$ L according to the RealMasterMix (SYBR Green I) protocol (Tiangen Corp.). Samples were assayed in triplicate and the data were analyzed by the method (Livak and Schmittgen, 2001). *GAPDH* was used as an endogenous reference to normalize the amount of cDNA used in the PCR and brown-shelled chickens were used as the calibrator. The amount of *HMOX1* transcripts, normalized to *GAPDH* and relative to the calibrator, was given by. The *HMOX1* forward and reverse primers were 5'-ATCGCATGAAAACAGTCCAG-3' and 5'-CAAATAAGCCCACGGCGAC-3', respectively, and the *GAPDH* forward and reverse primers were 5'-ATACACAGAGGACCAGGTTG-3' and 5'-AAACTCATTGTCATACCAGG-3', respectively.

### Western blotting

Total protein was extracted from individual frozen liver and spleen samples using 500  $\mu$ L of lysis buffer consisting of 7 M urea, 2 M thiourea, 60 mM dithiothreitol (DTT) and 4% 3-[(3-cholamidopropyl)-dimethylammonio]-propane-sulfonate (CHAPS). Protein from three individuals was pooled into a sample (with four pools or groups per eggshell color). Protein concentration was determined using a Bradford dye-binding protein assay kit (Jiancheng Bioengineering Institute, NanJing, China). Twenty micrograms of total protein were separated by SDS-PAGE on 10% gels followed by a transfer to a nitrocellulose mem-

brane (0.45  $\mu$ m) at 100 V for 1 h 20 min. The membrane was subsequently incubated in blocking buffer (3% BSA and 0.1% Tween 20) for 1 h at 37 °C and then for 1 h at 37 °C in blocking buffer containing rabbit anti-HO-1 antibody (dilution 1:200, Biosynthesis Biotechnology Co., Beijing, China) and  $\beta$ -actin antibody (dilution 1:2000, Biosynthesis Biotechnology Co.). After three washes in TBST (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.1% Tween 20) for 10 min each, the membrane was exposed to horseradish peroxidase-conjugated secondary antibodies at 37 °C for 1 h. After three washes in TBST, the bands were visualized by exposing the membrane to diaminobenzidine (DAB) for 3–5 min.

### HO activity assay

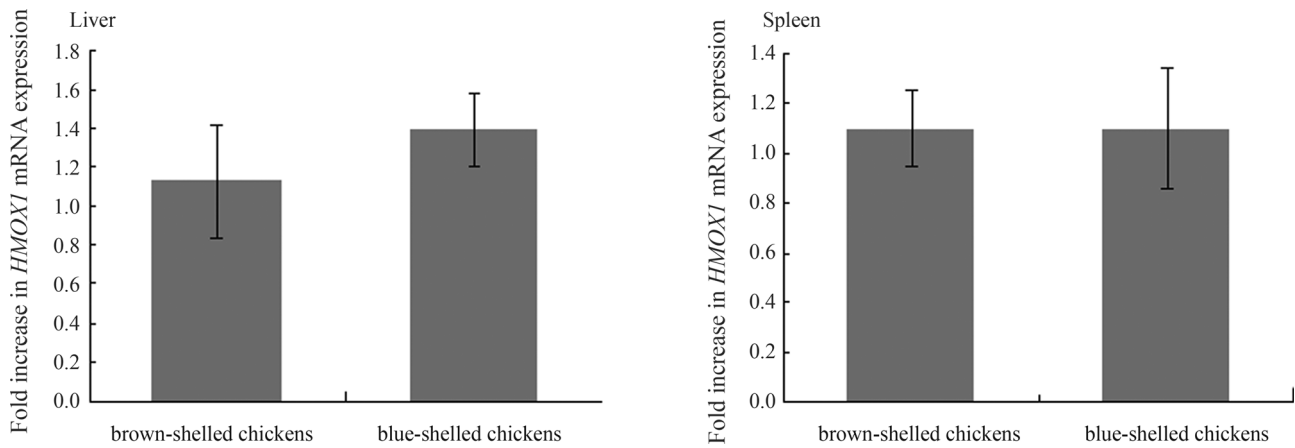
HO activities in liver and spleen were evaluated spectrophotometrically by measuring bilirubin formation (McCoubrey, 1999). Briefly, 15  $\mu$ L of fresh microsomes (protein concentration: 8 mg/ml) was added to a reaction mixture containing 6  $\mu$ L of 2.75 mM NADPH (substituted by HO assay buffer in the reference tube), 10  $\mu$ L of porcine kidney cytosolic fraction as a source of biliverdin reductase (0.9 U total), 26  $\mu$ L of HO assay buffer and 9  $\mu$ L of 1 mM hemin (substrate). The samples and reference tubes were incubated for 20 min at 37 °C in the dark with constant shaking. The reaction was stopped by placing the tubes in an ice bath. The solution in the reference tube was used as blank in the spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific Inc.) readings done between 410 nm and 610 nm. The difference in absorption between 470 nm and 530 nm was determined and the concentration of bilirubin was calculated using an extinction coefficient of 40 mM<sup>-1</sup> cm<sup>-1</sup>. One unit of HO activity was defined as the production of 1 nmol of bilirubin/h.

### Statistical analysis

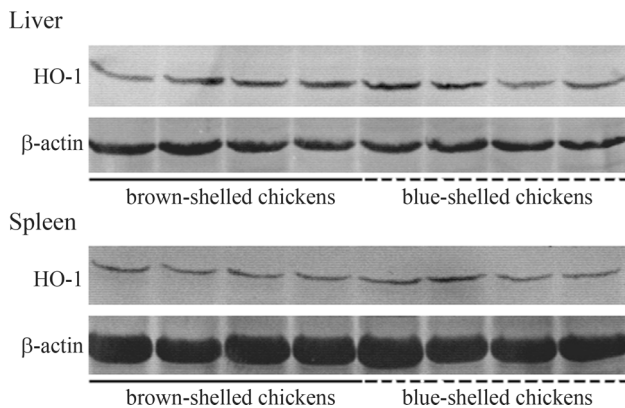
The results were expressed as the mean + SEM. Statistical comparisons were done using Students *t*-test with a value of  $p < 0.05$  indicating significance. All data analyses were done using the statistical software package SAS v.8.2.

## Results

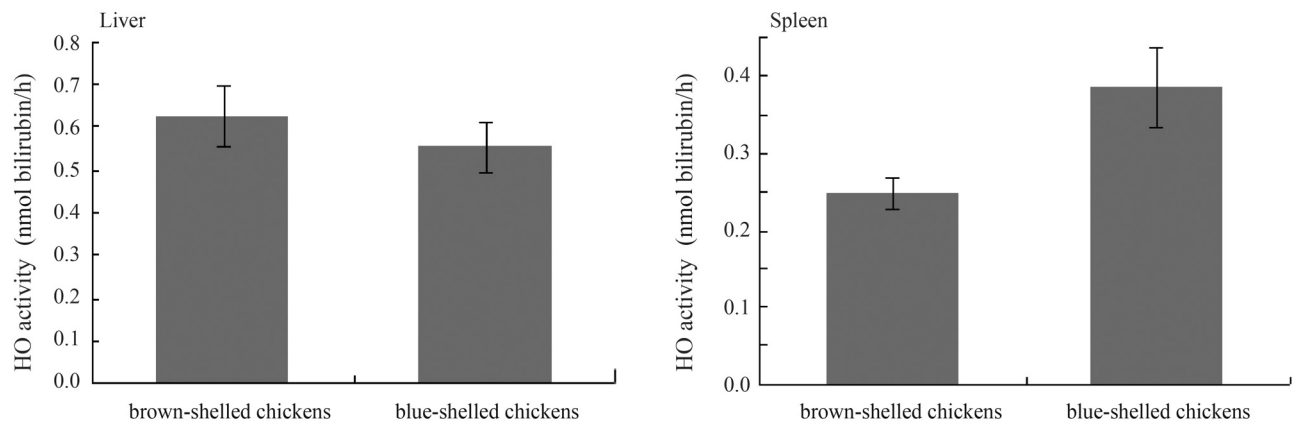
The liver and spleen are two tissues in which heme is oxidatively degraded to biliverdin, CO and Fe<sup>2+</sup>. As shown here, *HMOX1* expression and HO activity were detected in both of these tissues in blue-shelled and brown-shelled chickens. However, there was no significant difference in the *HMOX1* mRNA levels in liver or spleen in these two groups of chickens (Figure 1). In agreement with this, there was also no significant difference in the hepatic and splenic protein expression of HO-1 between the two groups, as assessed by western blotting (Figure 2). This finding was fur-



**Figure 1** - Quantitative real-time RT-PCR analysis of *HMOX1* expression in brown-shelled chickens and blue-shelled chickens. The bars represent the mean + SEM fold increase in *HMOX1* transcript expression relative to the brown-shelled group given by  $2^{-\Delta\Delta CT}$  ( $n = 12$  per group). Blue-shelled chickens expressed 1.39 fold more transcripts in liver than brown-shelled chickens, but the difference was not significant. There was also no significant difference in the level of spleen *HMOX1* transcripts in the two groups.



**Figure 2** - HO-1 protein expression. Protein extracts from three chickens in each group were pooled and each pool was assayed four times for HO-1 protein by western blotting. The four replicates are shown in the blots. The two groups of chickens expressed similar amounts of HO-1 protein in liver and spleen.



**Figure 3** - Hepatic and splenic HO activity. Enzymatic activity was assayed by measuring bilirubin formation. There were no significant differences in the hepatic and splenic HO activities between brown-shelled chickens and blue-shelled chickens. The columns are the mean + SEM of 12 chickens per group.

ther supported by a lack of difference in HO-1 activity between the two groups (Figure 3).

## Discussion

Blue is a common eggshell color in birds, not only in domestic fowls such as chicken (Punnett, 1933), duck (Wang *et al.*, 1997) and quail (Ito *et al.*, 1993), but also in wild birds such as eastern bluebird (Siefferman *et al.*, 2006), blue-footed booby (Morales *et al.*, 2010), and pied flycatcher (Moreno *et al.*, 2005). The blue color is considered to be a signal to males of a female's state of health such that males are more likely to remain around nests and help rear the young (Moreno and Osorno, 2003). The blue color derives from biliverdin, a breakdown product of heme. However, the site of synthesis of this biliverdin remains unresolved.

Previous studies have shown that the shell gland and eggshell of blue-shelled chickens contain higher levels of biliverdin than those of brown-shelled chickens (Zhao *et al.*, 2006; Wang *et al.*, 2009). These findings suggest that biliverdin is synthesized in the shell gland. Since biliverdin in bile is synthesized mainly in liver and spleen it is possible that biliverdin in eggshell may be derived from these tissues, being transported to the shell gland via the blood. To address this question, we examined the expression levels of *HMOX1* in liver and spleen. If *HMOX1* were highly expressed in these two tissues of blue-shelled chickens it would suggest a greater efficiency of biliverdin biosynthesis in these tissues, thereby providing additional evidence that eggshell biliverdin is synthesized outside the shell gland. However, our data indicate that there were no significant differences in HO-1 expression or activity in either tissue from the two groups of chickens (Figures 1-3). This finding agrees with that of Zhao *et al.* (2006) who also observed no significant difference in the bile biliverdin levels in these two groups of chickens. Together, these data indicate that there is insufficient bile biliverdin to escape from the enterohepatic circulation to the shell gland in blue-shelled chickens.

In laying hens, the shell gland is a highly vascular organ. When the egg reaches the shell gland, the metabolism of this organ is stimulated, blood flow increases (Kennedy and Vevers, 1973; Baird *et al.*, 1975) and protoporphyrin (the precursor of heme, another eggshell pigment responsible for red-brown eggshell color) is released during erythrocyte disintegration and is transported to the surface of the eggshell by wandering cells (Baird *et al.*, 1975). Based on current knowledge of the process of protoporphyrin deposition it is conceivable that heme resultant from erythrocyte degradation can be converted to biliverdin by HO-1 in the shell gland of blue-shelled chickens since this organ has a high expression of *HMOX1* (Wang *et al.*, 2010, 2011) and would result in blue-colored eggs.

In conclusion, our data support the hypothesis that eggshell biliverdin is synthesized in the shell gland. Although this study and previous reports (Wang *et al.*, 2010, 2011) have focused on *HMOX1*, a gene associated with the biosynthesis of biliverdin, future studies should examine the mechanism by which biliverdin is transported.

## Acknowledgments

The authors thank Xu JianSheng and Chen YunLong for providing samples. This work was funded by the Northwest Scientific Startup Foundation for Doctors (Z109021112), the National Nature Science Foundation (31072024) and the Lueyang Chicken Breeding Project of Northwest A&F University (Z109021127).

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*Associate Editor: Alexandre Rodrigues Caetano*

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