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Quantification and variability of eggshell pigment content

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A high-performance liquid chromatography method was used for the determination of the eggshell pigment constituents. Using this method, the content protoporphyrin IX in seven bird species was quantified. It was found that this compound is most abundant in the eggs of the blackbird (65.55 nmol per egg), but when the amount is related to the eggshell mass, then the greatest amount is in the egg of the yellowhammer (301.0 pmol/mg eggshell), while lowest values were found in the eggs of the swift (0.59 nmol); dunnock egg shells also contained biliverdin (10.25 nmol). Eggs of the red-backed shrike show a high variability of porphyrin content (9.40 ± 6.27 nmol; range 1.03–20.61 nmol per egg). The high intra-species variability of the porphyrin content is likely to reflect physiological influences, e.g. order of egg laying and nesting conditions rather than the effect of the environment.

Key words: Eggshell; Pigment; Porphyrin; Protoporphyrin; Biliverdin; Bird; Red-backed shrike; High-performance liquid chromatography (HPLC); Environment.

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Introduction

As published by Kennedy and Vevers (1973, 1976), eggshell pigmentation reflects the presence of porphyrins in the eggshell. This pigmentation seems to have preferably cryptic reasons; the demand for minimum solar heating of the eggs is unlikely to play an important role in coloration as studies on spectral reflectance proved that differently coloured eggs exhibit uniformly high reflectance in the near infra-red region, independently of the eggshell colour (Bakken *et al.*, 1978). The present knowledge about eggshell coloration is rather limited, as the published information does not involve any quantitative data or data about variability of their content. As it is known that coloration differences of eggshell are considerable, it seemed interesting to investigate the differences in the composition and pigment content of differently coloured eggshells.

Changes in eggshell coloration may reflect physiological condition like egg laying or nesting, but they may result from exogenous (environmental) factors as well. Information regarding changes in porphyrin content and metabolism in liver as the result of halogenated aromatic hydrocarbons exposure is well documented. As a result of this type of poisoning, the elevated concentration of highly carboxylated porphyrins in the herring gulls liver from the Great Lakes was

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described by Fox *et al.* (1988). In concert with this finding are the results by Miranda *et al.* (1983) and Elliott *et al.* (1990) who were able to demonstrate increased porphyrin content in livers of the Japanese quail, but in the American kestrel, the exposure to polychlorinated biphenyls remained without any effect (Elliott *et al.*, 1991). On the other hand, exposure to hexachlorobenzene and tetrachlorohydroquinone results in pronounced porphyria in the Japanese quail (Carpenter *et al.*, 1985). It was also demonstrated (Kawanishi *et al.*, 1981) that exposure to polychlorobiphenyls results in the inhibition of uroporphyrinogen decarboxylase in cultured chick embryo liver cells. To our best knowledge, no information about the quantitation of the pigment composition of differently coloured eggs is available from the literature. Also, our literary search for data about eggshell coloration changes as the result of environmental factors was unsuccessful.

The purpose of this work is to elaborate a simple method for eggshell porphyrin analysis, to compare the eggshell pigment composition in different species and to evaluate whether or not the environmental factors play a role in eggshell pigmentation.

Materials and Methods

Mainly non-hatched eggs from reared nests (i.e. successful nesting) were used in this investigation; in the case of the red-backed shrike (*Lanius collurio*), also one unsuccessful clutch (four eggs) was analysed.

A porphyrin ester chromatographic kit was obtained from Porphyrin Products (Logan, UT, U.S.A.), protoporphyrin IX, protoporphyrin IX dimethyl ester and biliverdin from Sigma (St. Louis, MO, U.S.A.) and hematoporphyrin IX from Aldrich (Milwaukee, WI, U.S.A.).

Preparation of porphyrins from eggshells (as methyl esters) was done by the procedure described by Kennedy and Vevers (1973). Egg shells were cleaned and washed by distilled water (MilliQ) and then solubilized (and esterified) in 30 ml absolute methanol containing 5% concentrated sulphuric acid at room temperature in the dark for 2 days. After this period, the coloured extract was filtered (to remove shell membranes) and 15 ml chloroform and 10 ml distilled water were added with shaking. The colourless epiphase was discarded, and the coloured hypophase was washed with 10 ml 10% NaCl, followed by distilled water, until the washings were neutral. The extract was evaporated to dryness and reconstituted in 1 ml chloroform. Validation of the procedure was done by esterification of standards following the same protocol.

Porphyrins were analysed by reversed-phase high-performance chromatography using Waters automated gradient controller (Millipore, Milford, MA, U.S.A.), Waters Model 510 pumps and steel 300 × 3.9 mm PicoTag column (C18). The sample (30 μl) was injected into the column and eluted with a gradient consisting of (A) methanol/distilled water/pyridine 35:65:0.25 v/v and (B) methanol/acetonitrile/pyridine 90:10:0.25 v/v (flow rate 1.2 ml/min and temperature 55°C). The gradient started at A/B 80:20 reaching 10:90 ratio after 15 min. During the next 10 min, the elution was isocratic followed by another 10 min isocratic elution at an A:B ratio of 5:95. Elution was monitored by absorbance at 410 nm (Waters 490E multiwavelength detector) and by fluorescence at $405_{ex}/620_{em}$nm (Fluorescence monitor RF-530, Shimadzu, Kyoto, Japan).

Results and Discussion

The chromatographic system used allowed for baseline separation of methyl esters of porphyrins with retention times summarized in Table 1. All porphyrins were detected simultaneously by absorbance (410 nm) as at fluorescence ($405_{ex}/620_{em}$nm).

Table 1. Retention times of methyl esters of porphyrins by reversed-phase chromatography

Methyl ester porphyrin	Retention time (min)
8-Carboxyporphyrin (uroporphyrin I)	15.1
7-Carboxyporphyrin	15.9
6-Carboxyporphyrin	16.7
5-Carboxyporphyrin	17.5
4-Carboxyporphyrin (coproporphyrin I)	18.3
Mesoporphyrin IX	22.0
Protoporphyrin IX	23.9
Hematoporphyrin IX	13.4
Biliverdin	15.9

Table 2. Content (absolute and relative) of protoporphyrin IX and biliverdin in eggshells of various bird species

Species	Protoporphyrin IX nmol	Protoporphyrin IX pmol/mg eggshell	Biliverdin nmol	Biliverdin pmol/mg eggshell
Blackbird (*Turdus merula*)	65.55	152.4	—	—
Yellowhammer (*Emberiza citrinella*)	42.14	301.0	—	—
Red-backed shrike* (*Lanius collurio*)	9.40	52.1	—	—
Crested tit (*Parus cristatus*)	5.17	73.9	—	—
House sparrow (*Passer montanus*)	4.87	48.4	—	—
Dunnock (*Prunella modularis*)	0.62	6.2	10.25	102.5
Swift (*Apus apus*)	0.59	2.9	—	—

*Average from 22 eggs.

biliverdin was detected only by absorbance (also 410 nm), because it could not be detected at selected fluorescence parameters. Calibration was linear in the range 0.015–0.900 nmol/injection, $r = 0.9996$ with the detection limit 0.003 nmol for all porphyrins (inclusive protoporphyrin); with biliverdin, however, the calibration was linear in the range 0.060–0.450 nmol/injection, $r = 0.9998$ with the detection limit 0.020 nmol.

Egg porphyrins of seven bird species were monitored (blackbird—*Turdus merula*, yellowhammer—*Emberiza citrinella*, crested tit—*Parus cristatus*, house sparrow—*Passer montanus*, swift—*Apus apus*, dunnock—*Prunella modularis* and red-backed shrike—*Lanius collurio*). In all species investigated, the pigments contained protoporphyrin (and in one case also biliverdin) only. These results are in agreement with the findings of Kennedy and Vevers (1975) who examined 108 bird species. On the contrary, With (1973) found a mixture of porphyrins (in addition to protoporphyrin, also coproporphyrin, pentacarboxylic porphyrin, uroporphyrin and some unidentified porphyrins) in brown hen's eggs. The findings of additional porphyrins in this case may reflect the difference between artificial breeding and wild living birds. For example, Kennedy and Vevers (1973) found also traces of coproporphyrin in the eggshells of the Araucano fowl.

The amounts of protoporphyrin found are summarized in Table 2. It is obvious that the amount of porphyrin found depends on the colour of the egg. All non-uniformly coloured bird eggs are variable in their colour appearance. The eggs of the blackbird are darker, light blue with brownish markings; yellowhammer eggs are white with spotting and scribbling in pale purple–grey or reddish purple with irregular scrawls and small blotches in black or purplish brown; red-backed shrike eggs are very varibly white, creamy or pinking usually with a distinct zone of brown, grey and chestnut-red spots and small blotches forming a band around the larger end; the eggs of the crested tit are white, spotted and finely blotched with purplish red; house sparrow eggs are white or greyish variably marked by blotches of grey or brown; dunnock eggs are uniformly bright blue and swift eggs are white. Blackbird, yellowhammer, red-backed shrike and dunnocks have their nests in bushes or trees and crested tit and house sparrow have their eggs in holes, while swift nests are usually in buildings under eaves.

The detailed study about variability of the proporphyrin IX content in eggshells was made on the eggs of the red-backed shrike (*Lanius collurio*). The detected amount was highly different (see Fig. 1). The mean value for all measured eggs was 9.40 ± 6.27 nmol per egg ($n = 22$) (range 1.03–20.61); when the amount of protoporphyrin is related to the eggshell mass, then

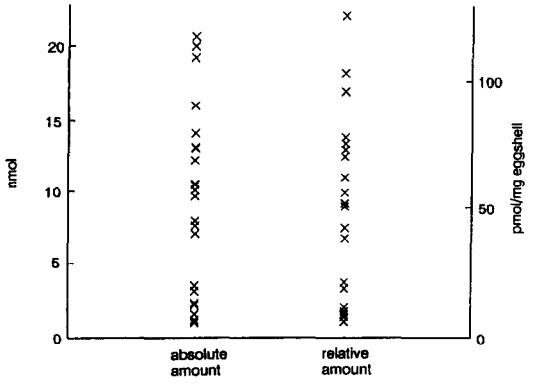

Fig. 1. The amount of protoporphyrin IX detected in the eggshell of red-backed shrike (absolute amount—nmol per egg; relative amount—pmol/mg eggshell).

the value is 52.1 ± 35.1 pmol/mg eggshell (range 6.2–124.9). When the eggs were divided into the two groups, non-hatched eggs in successful nests and eggs in unsuccessful nests, the values were not significantly different (10.41 ± 6.71 nmol, $n = 12$ vs. 9.03 ± 8.60 nmol per egg, $n = 4$). Also, the range within which the individual values varied were quite similar (1.03–20.61 nmol vs. 1.61–19.21 nmol per egg). These results indicate that the content of porphyrin is independent of the possible variable abrasion of eggs due on the time of brooding of the eggs.

The high differences in eggshell colouring reflect the differences in the content of protoporphyrin (with the exception of the dunnock where biliverdin contributes to the final colouring as well). Because of this variability in porphyrin content, it is impossible to reach small values of standard deviation in the analyses. Data on the whole clutch of shrike (1.61, 2.21, 13.09 and 19.21 nmol) suggest that the porphyrin content may correspond with the order of egg laying (decreasing towards the last egg). The data in Fig. 1 are indicative of this conclusion. Owing to the short period elapsed between egg laying (usually 1 day), this variability is unlikely to be caused by exogenous (environmental) factors.

It is possible to assume that high intraspecies variability of porphyrin content probably reflects influences other (e.g. order of egg laying and whole condition of the nesting female) than the influence of the environment.

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