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Avian Eggshell Pigments and Their Variability

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ABSTRACT. Eggshell pigment constituents were determined by a high-performance liquid chromatography method. Most of the work was done on whole clutches of red-backed shrike (*Lanius collurio*). In addition to the known pigments (protoporphyrin IX, biliverdin), a new pigment, zinc-containing protoporphyrin IX, was found as well. Its content was highly variable—from 0% to 100%. The content of total protoporphyrin IX (with and without zinc) ranged from 1.72 to 114.84 nmol (average for whole clutches was 39.93 nmol), while the content of zinc-deficient protoporphyrin IX was ND (not detectable) −110.46 nmol (average for whole clutches was 34.02 nmol) and the content of zinc-containing protoporphyrin IX was *ND* −32.28 nmol (average for whole clutches was 5.91 nmol). Zinc-containing protoporphyrin IX was absent in 33% of eggs, while zinc-deficient protoporphyrin IX was absent in a single case only (2%). If clutches from relatively "polluted" and "unpolluted" regions were compared, no differences have been found. Based on these findings is the conclusion that the high variability in eggshell pigment content is likely to reflect physiological influences (e.g. order of egg laying and the whole condition of the nesting female) rather than environmental interferences. This conclusion is in agreement with our previous findings. Additional data regarding the pigment content of seven other bird species are also included. COMP BIOCHEM PHYSIOL 113B, 607–612, 1996.

KEY WORDS. Eggshell, pigment, porphyrin, metalloporphyrin, protoporphyrin, biliverdin, red-backed shrike, environment

INTRODUCTION

Avian eggshell is covered by a pigmented layer that may cap or plug the pore canals. Board and Scott (2) use the term "shell accessory material" (SAM) for all layers occurring on the outer surface of the calcitic shell. This layer has a basic task in maintaining the integrity of the shell's gaseous diffusion pathway against obstruction by nest debris, flooding with water and in reducing the probability of bacterial penetration of the shell post-oviposition. Inorganic SAM is composed mainly of vaterite (one of the four polymorphs of calcium carbonate, i.e. calcite, aragonite, vaterite and amorphous calcium carbonate) (21). Colour of SAM results from the presence of eggshell porphyrin pigments. Kennedy and Vevers (13,14) discovered that brown and black pigments are associated with protoporphyrin IX while blue or green colour is associated with biliverdin IX and biliverdin zinc chelate. Sparks (21) reviewed that pigments occur primarily in the cuticle but can be detected in the mineralized shell immediately opposed to the cuticle or may be located wholly or partially in the underlying shell. 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 infrared region, independent of the eggshell colour (1).

The eggshell pigment is accumulated, in the case of quail, in the shell gland after ovulation time and deposited on the surface of eggshell 3.5 to 2 hr before oviposition. Steroid hormones (progesterone) may affect the accumulation of pigment in the quail shell gland (19).

Eggshell colour may considerably differ and so it seems interesting to investigate the differences in the composition and pigment content of differently coloured eggshells. As concluded in our previous work (16), changes in eggshell coloration may reflect physiological condition like egg laying or nesting, but they may possibly result from exogenous (environmental) factors as well. It is well documented in the literature that changes in the porphyrin content and its metabolism in liver result from the exposure to halogenated aromatic hydrocarbons. Halogenated aromatic hydrocarbons have been shown to elevate the level of porphyrins in the livers of Herring gulls (8) and Japanese quail (4,17). On the other hand, however, in American kestrel the exposure to polychlorinated biphenyls remained without any effect (5), though exposure of Japanese quail to hexachlorobenzene and tetrachlorohydro-

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quinone resulted in pronounced porphyria (3). It was also demonstrated (12) that exposure to polychlorobiphenyls results in the inhibition of uroporphyrinogen decarboxylase in cultured chick embryo liver cells. The influence of organochlorines on birds is, of course, more complex. Their role in eggshell thinning is well documented (see e.g. recent papers of Elliott and Martin; Forsyth *et al.*; Lundholm) (6,7,15).

To the best of our knowledge very limited 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 colouration changes as the result of environmental factors was unsuccessful.

In this study we investigated mainly eggshell pigments of red-backed shrike (*Lanius collurio*). This species is now declining throughout Europe (as are other shrikes) and, consequently, is under intensive investigation. One of the crucial factors in this decline is probably environmental conditions. Shrike's eggs are very variable in colour. Therefore we tried to determine the pigments in shrike's eggshells with our aim to discern whether or not the environmental factors play a role in eggshell pigmentation.

MATERIALS AND METHODS

Mainly eggs of unsuccessful clutches (i.e. abandoned nests) of red-backed shrike (*Lanius collurio*) were used in this study. These eggs were collected in two regions of the Czech Republic: in Prague (polluted capital of the state) and in Vsetín region (relatively unpolluted locality).

All porphyrins were in the form of dimethyl ester. Standards of protoporphyrin IX and biliverdin (from Sigma, St Louis, MO, U.S.A.) were handled in the same way as samples. The porphyrin ester chromatographic kit was obtained from Porphyrin Products (Logan, UT, U.S.A.).

Metal porphyrins were prepared according to Fuhrhop and Smith (9). Briefly, porphyrin in chloroform was mixed with a saturated solution of metal acetate in methanol followed by a few minutes of refluxing. The mixture of evaporated and reconstituted in chloroform. Zinc biliverdin was also prepared by the method of Kennedy and Vevers (13) by boiling the ethanolic solution of biliverdin with zinc powder.

Preparation of porphyrins from eggshells (as methyl esters) was done by the procedure described by Kennedy and Vevers (13). Egg shells were cleaned and washed by distilled water (Milli-Q) and then solubilized (and esterified) in 30 ml absolute methanol (LiChrosolv, gradient grade for chromatography, Merck, Darmstadt, Germany) 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 (Merck, Chloroform GR, ISO, certified Zn max. 0.00001%) and 10 ml distilled water were added, then shaken. The colourless epiphase was discarded and 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.

Absorption spectra were measured by PU 8740 UV/VIS Scanning Spectrophotometer (Philips, Eidhoven, The Netherlands).

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 (50 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. For the next 10 min the elution was isocratic followed by another 10 min isocratic elution at 100% B. 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). Biliverdin, on the contrary to other porphyrins, can not be detected by selected fluorescence and is accessible by absorbance detection only. Therefore, comparing fluorescence and absorbance detection may help identification of this porphyrin.

The used chromatographic system was successful in separation of methyl esters of porphyrins and their metal complexes (see Table 1). Calibration was linear in the range 0.01–10 nmol/injection with the limit of detection 0.003 nmol for all porphyrins (for zinc-deficient protoporphyrin $r = 0.989$ and for zinc-containing protoporphyrin $r = 0.991$); with biliverdin, however, the calibration was linear in the range 0.05–2 nmol/injection, $r = 0.991$ with the limit of detection 0.020 nmol.

Thin-layer high-performance chromatography was carried out on HPTLC plates RP-18 F_{254}s (10 × 10 cm) for nano TLC (Merck). The compounds were detected as quenching spots under UV light at 254 nm or as fluorescent spots under UV light at 366 nm (Min UVIS; Desaga, Heidelberg, Ger-

TABLE 1. Retention times of methyl esters of porphyrins without or with Zn^{2+} by reversed-phase liquid chromatography

Methyl ester porphyrin	Retention time (min)	
	Without Zn^{2+}	With Zn^{2+}
8-Carboxyporphyrin (uroporphyrin I)	15.3	14.7
7-Carboxyporphyrin	16.0	15.4
6-Carboxyporphyrin	16.8	16.0
5-Carboxyporphyrin	17.6	16.7
4-Carboxyporphyrin (coproporphyrin I)	18.5	17.4
Mesoporphyrin IX	22.2	19.7
Protoporphyrin IX	23.9	20.0
Biliverdin	16.0	15.8

many). Plates were developed with pure ethanol or methanol. In the case of ethanol, the relative mobility ($R_F \times 100$) for Zn^{2+}-deficient protoporphyrin was 18; Zn^{2+}-containing protoporphyrin, 49; Zn^{2+}-deficient biliverdin, 64; and Zn^{2+}-containing biliverdin, 69; in the case of methanol, mobility for Zn^{2+}-deficient protoporphyrin was 12; Zn^{2+}-containing protoporphyrin was 38; Zn^{2+}-deficient biliverdin was 54; and Zn^{2+}-containing biliverdin was 59.

RESULTS

A new porphyrin eggshell pigment, zinc-containing protoporphyrin IX, was found in some preparations. Identification was made by comparing the absorption spectrum and retention times in high-performance liquid chromatography and high-performance thin-layer chromatographic mobility with an appropriate standard. Absorption spectrum in chloroform of standard zinc-containing protoporphyrin IX gave maximum at 416, 544 and 581 nm and this spectrum was identical with spectra obtained i) after the reaction of extracted protoporphyrins from egg with zinc acetate and ii) with the egg extract that contained probably only this metalloporphyrin. Analogous results were obtained by both chromatographic methods. Retention time (or R_F in TLC) was identical for Zn^{2+} protoporphyrin IX and for the product arising after the reaction of extracted protoporphyrins from egg with zinc acetate (i) and for the extracted material of the egg, which contained probably only this metalloporphyrin (ii). This metalloporphyrin can't arise from impurities present due to the extraction process, because the standard of zinc-deficient protoporphyrin IX prepared by the identical procedure from eggshells didn't give any peak with retention resembling the Zn complex. Based on these observations it is feasible to conclude that zinc-containing protoporphyrin IX occurs in the eggshells. In the next stage of our investigation we attempted to quantify this new pigment.

Observed retention times of porphyrins and zinc-containing porphyrins are summarized in Table 1. Protoporphyrin IX was examined also as Cu^{2+} complex. The retention time of Zn-containing protoporphyrin IX upon HPLC was 19.9 min, Zn-deficient protoporphyrin IX was 23.9 min and Cu-containing protoporphyrin IX was 32.0 min. In all investigated eggshells the Cu^{2+} complex was not found.

The content of total-PP (i.e. sum of protoporphyrin IX with and without zinc) (Fig. 1C) and in the form of zinc-deficient PP (Fig. 1A) and zinc-containing PP (Fig. 1B) is highly variable. This broad span is seen both as inter-clutch and as intra-clutch variations. Clutch average (clutch size was 5–6 eggs) of total PP (n = 9) was 39.93 ± 12.09 nmol (16.20–54.66 nmol); for zinc-deficient PP the clutches' average was 34.02 ± 10.78 nmol (15.81–51.01 nmol); and the value for zinc-containing PP was 5.91 ± 4.57 nmol (0.38–14.95 nmol). When investigating individual eggs (n = 49), the span of values was, of course, higher for the total PP; 1.72–81.81 nmol (average 39.88 ± 18.70 nmol); for zinc-deficient PP *ND* (not detectable)–75.66 nmol (average 33.74 ± 18.63 nmol); and for zinc-containing PP *ND*–32.28 nmol (average 6.15 ± 8.37 nmol). Zinc-deficient PP wasn't found in one case only, while zinc-containing PP was absent 16 times (i.e. 32.7%). The average proportion of zinc-containing PP to total PP in clutches was 16.54% (3.75–35.45%); in the case of individual eggs the ratio was 17.32% (0.00–100.00%).

The influence of environmental factors on the pigment content was studied by comparison of the total of clutches from Prague (n = 5) and Vsetín region (n = 4) (for details about environment see Discussion). In these sets we didn't discover any significant difference. The average for total PP was 41.42 *vs.* 38.07 nmol; for zinc-deficient, PP was 36.14 *vs.* 31.37 nmol; for zinc-containing, PP was 5.28 *vs.* 6.69 nmol and the proportion of zinc-containing PP to total PP was 15.97 *vs.* 17.25%. In principle the same result was obtained when averages for the individual eggs were compared (examined clutches contained 5–6 eggs): total PP 40.89 *vs.* 38.55 nmol, zinc-deficient PP 35.44 *vs.* 31.46 nmol, zinc-containing PP 5.44 *vs.* 7.09 nmol and the proportion of zinc-containing PP to total PP was 16.72 *vs.* 18.12%.

The span of protoporphyrin content in the case of red-backed shrike was larger. When we added some individual non-hatched eggs (four from one nest and three times one egg), the average of total PP was 46.03 ± 24.69 nmol (span was 1.72–114.84 nmol); zinc-deficient PP was 40.05 ± 25.01 nmol (span was *ND*–110.46 nmol); zinc-containing PP was 5.98 ± 8.05 nmol (span was *ND*—32.28 nmol) and the proportion of zinc-containing PP to total PP was 15.84 ± 23.36% (0.00–100.00%).

All these values (i.e. the high span and SD) indicate a high variability of porphyrin content in eggshells of shrike. This is in agreement with the variability of shrike eggshell color. In connection with these observations one may ask the question about pigment content variability in other bird species. Preliminary data in this respect are summarized in Table 2. It is obvious that the amount of porphyrin found depends on the colour of the eggs (from white eggs of starling to the highly spotted eggs of yellowhammer).

Although the data regarding variability of pigment content in Table 2 are rather limited, it is feasible to assume that the span of values in all investigated species is high. Variability of shrike eggshell colouration is the highest, which is in agreement with span of pigment content.

DISCUSSION

Our results about protoporphyrin IX and biliverdin are in agreement with the findings of Kennedy and Vevers (14) who examined 108 wild bird species. In the eggshell of the Araucano fowl they also found traces of coproporphyrin (13). With (23) found in brown hen's eggs a mixture of porphyrins (in addition to protoporphyrin, also coproporphyrin, pentacarboxylic porphyrin, uroporphyrin and some unidentified porphyrins). These findings may reflect the difference between

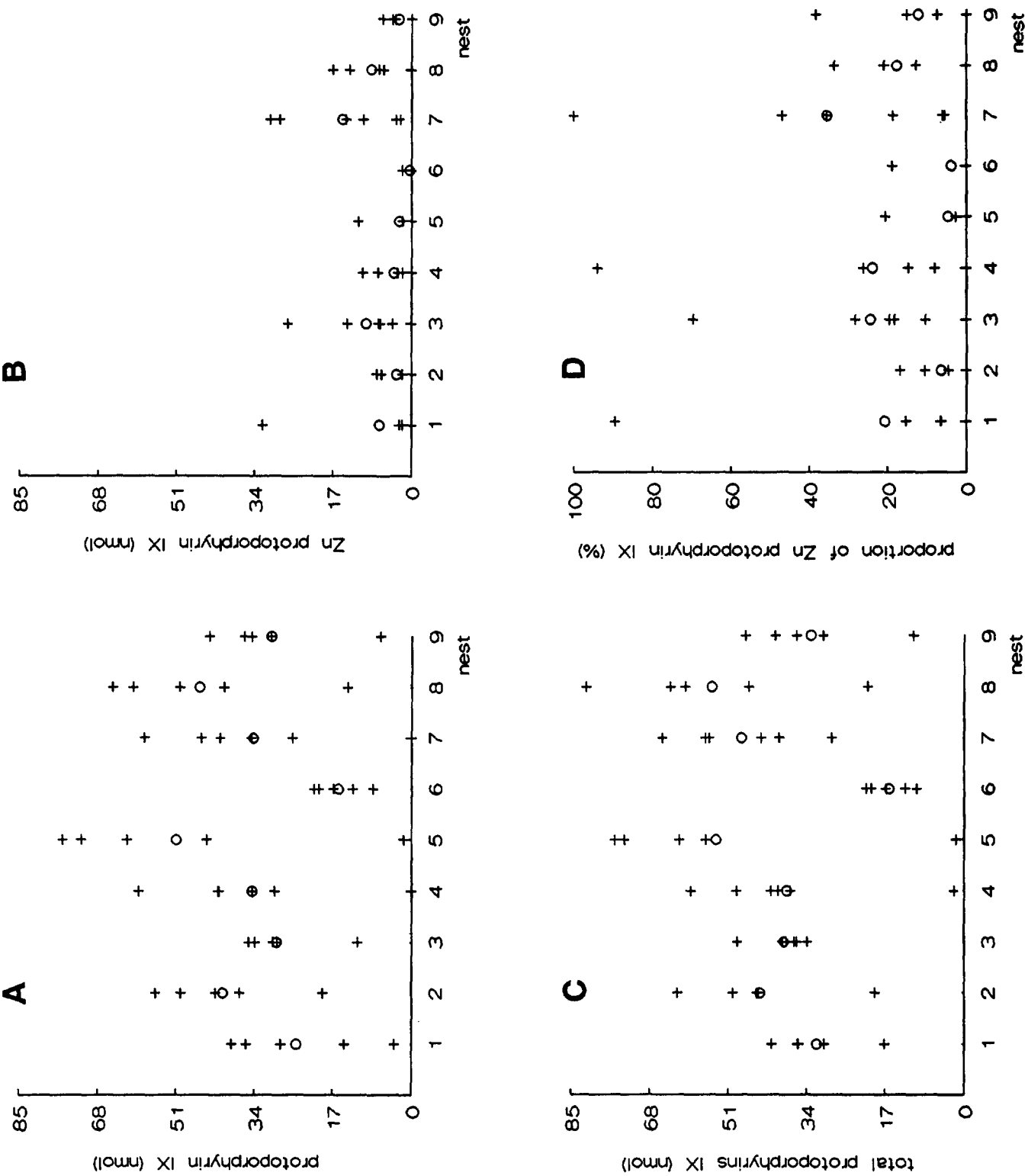
A
protoporphyrin IX (nmol)
B
Zn protoporphyrin IX (nmol)
C
total protoporphyrins IX (nmol)
D
proportion of Zn protoporphyrin IX (%)
nest
0
17
34
51
68
85
100
80
60
40
20
1
2
3
4
5
6
7
8
9

TABLE 2. Summary of individual pigment content in various bird species (in nmol)

Species[a]	Protoporphyrin IX			Biliverdin
	Total	Without Zn^{2+}	With Zn^{2+}	
Yellowhammer (*Emberiza citrinella*)	224.08	220.16	3.92	—[b]
	223.83	219.84	3.99	—[b]
	220.22	215.79	4.43	—[b]
	169.12	165.71	3.41	—[b]
Blackbird (*Turdus merula*)	119.33	116.76	2.57	—[b]
	105.38	103.52	1.86	—[b]
Barred warbler (*Sylvia nisoria*)	80.29	75.03	5.26	—[b]
	41.55	37.04	4.51	—[b]
Chiffchaff (*Phylloscopus collybita*)	38.29	35.85	2.44	—[b]
	12.28	12.28	—[b]	—[b]
Dunnock (*Prunella modularis*)	4.36	2.18	2.18	21.57
	2.22	2.22	—[b]	31.45
Black redstart (*Phoenicurus ochruros*)	7.37	7.37	—[b]	—[b]
	2.46	2.46	—[b]	—[b]
	1.28	1.28	—[b]	—[b]
	0.72	0.72	—[b]	—[b]
Starling (*Sturnus vulgaris*)	0.40	0.40	—[b]	4.71
	0.24	0.24	—[b]	6.04

[a]Values for individual eggs.
[b]Not detectable.

artificial breeding and wild living birds. Kennedy and Vevers (13,14) also found biliverdin zinc chelate in some bird species. In our egg collection, this pigment was not detectable.

The new pigment, zinc-containing protoporphyrin, was found in most species (except black redstart and starling). Porphyrin content was, as in the case of shrike, highly variable; in the case of chiffchaff and dunnock it was present in one egg, while in one egg absent. The discovery of this metalloporphyrin among eggshell pigments calls attention to the possibilities of influencing of eggshell colour by metals present in the environment through forming porphyrin chelates. This possibility, however, wasn't confirmed. The effort to discover environmental influences was realised in two regions—Prague and Vsetín. From these regions some environmental data from years 1993/94 are available (10,20). The average concentration (per year) for SO_2 was 30 $\mu g.m^{-3}$; for NO_x it was 40 $\mu g.m^{-3}$; and for dust aerosol it was 60 $\mu g.m^{-3}$ in the studied Prague area; while in the Vsetín region the values were about one half or one third. These results, however, were distorted by non-homogenous placing of the monitoring stations in the Vsetín region (differences between monitored towns and studied country areas). Average amounts of some metals in dust aerosols measured at Prague by monitoring station close to the study area (20) were: Pb, 50 $ng.m^{-3}$; Cd, 8 $ng.m^{-3}$; Zn, 190 $ng.m^{-3}$; Ni, 325 $ng.m^{-3}$; Cr, 2.3 $ng.m^{-3}$ and As, 0.8 $ng.m^{-3}$, which may be quite informative. Prague is classified as an ecologically affected region with high load, while Vsetín region is without any classification (10).

Literature about environmental pollutants presented in birds living in the Czech Republic is rather limited. There are very few data about chlorinated pesticides and heavy metals, e.g. in the eggs of water birds (11) or in the eggs of the black-headed gull (18). The results are very variable; the sum of chlorinated pesticides for clutches (great crested grebe, greylag goose, mallard, coot and black-headed gull) varied from 0.098 to 17.535 $mg.kg^{-1}$ in dry matter of eggs and in the case of polychlorinated biphenyls from 0.017 to 25.032 $mg.kg^{-1}$. Values of heavy metals in eggs were found: Cu, 0.450–4.530 $mg.kg^{-1}$ in dry matter of eggs; Hg, 0.009–0.365 $mg.kg^{-1}$; Pb, <0.100–0.720 $mg.kg^{-1}$; Cd, 0.010–0.160 $mg.kg^{-1}$; and Cr, <0.005–0.560 $mg.kg^{-1}$ (11).

It may be argued that for a study of environmental factors influencing the eggshell pigmentation, the controlled laboratory conditions would give better and more easily interpretable results. This, of course, may be another approach to the study of this problem, however one has to bear in mind the synergistic influences of the environment. This actually was the reason for proper field experiments. On the other hand it is true that the influences are, under such conditions, highly complex and the results may be difficult to interpret. This may be documented by the extensive work of Taper *et al.* (22) who studied 59 American songbird species in 22 regions differing

FIG. 1. Analysis of protoporphyrin content of red-backed shrikes' whole clutches. (A) Zinc-deficient protoporphyrin IX. (B) Zinc-containing protoporphyrin IX. (C) Total protoporphyrin IX. (D) Proportion of zinc-containing protoporphyrin IX to total protoporphyrin IX (+ individual value, ○ average of whole clutch). Nest numbers 1–5 are from Prague and nests 6–9 are from Vsetín region.

considerably in their characteristics over 20 years. No univocal conclusion from these data can be drawn.

It may be concluded that zinc-containing protoporphyrin IX is another member of the porphyrin (metalloporphyrin) family present in eggshells of the red-backed shrike. Its content is variable between single eggs (even in one clutch). When evaluating the influence of the environment, no differences were observed between eggs from polluted and unpolluted areas. In agreement with our previous result (16), it appears that the porphyrin content probably reflects other influences than environmental conditions (e.g. order of egg laying and whole condition of the nesting female).

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