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Analysis of Siamese Crocodile (*Crocodylus siamensis*) Eggshell Proteome

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Abstract
The proteins and pigment of the eggshell of the Siamese crocodile (*Crocodylus siamensis*) were analysed. For proteomic analysis, various decalcification methods were used when the two main surface layers were analyzed. These layers are important for antimicrobial defense of egg (particularly the cuticle). We found 58 proteins in both layers, of which 4 were specific for the cuticle and 26 for the palisade (honeycomb) layer. Substantial differences between proteins in the eggshell of crocodile and previously described birds' eggshells exist (both in terms of quality and quantity), however, the entire proteome of *Crocodilians* has not been described yet. The most abundant protein was thyroglobulin. The role of determined proteins in the eggshell of the Siamese crocodile is discussed. For the first time, the presence of porphyrin pigment is reported in a crocodilian eggshell, albeit in a small amount (about 2 to 3 orders of magnitude lower than white avian eggs).

Keywords Proteins of eggshell · Eggshell pigment · Crocodile

1 Introduction

The eggshell is an important structure, with calcareous eggs being produced by all birds and some reptiles. The eggshell is an important defense that protects the egg against microbial and other infections; it also guards the developing embryo against unfavorable impacts of the environment, and is essential for the reproduction of birds and many reptiles.

The majority of research and knowledge about eggshells has been obtained from studies on birds. This is understandable, because birds attract curiosity of many people and because birds' eggs are important for agriculture and human nutrition (and hence commercially important).

The avian eggshell is a complex structure formed during the movement of the egg along the oviduct by production of a multilayered mineral-organic composite [1]. Avian eggshells have a relatively simple structure: the outermost layer is a relatively thin cuticle, followed by a thick calcified layer composed of calcite, which forms crystalline structures termed "palisades", which are terminated by rounded cones, named "mammillae". The tips of the mammillary cones serve as anchor points with the fibres of the shell membranes that envelop the albumen [2].

The eggshell of the order of *Crocodilians* is well described for *Alligator mississippiensis* [3]. The thickness of the eggshell is 0.5–1.0 mm (including the inner shell membrane) and the eggshell can be divided into five layers: the outermost layer is a densely calcified layer (100–200 µm thick), then comes a honeycomb layer (300–400 µm thick), an organic layer (8–12 µm thick), a mammillary layer (20–29 µm thick), and the shell membrane (150–250 µm thick) [3]. The outer densely calcified layer has a granular nature because of its numerous calcite crystals. The honeycomb layer is porous and its fibrous organic matrix can be observed. The holes in this structure are similar to those of the palisade layer of the avian eggshell. The next (and thin) organic layer probably serves as a template for crystal growth. From the third week of incubation, the number of small calcite crystals in the organic layer decreases, so it appears that these crystals are mobilized for embryonic calcification [3].

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Shell formation in reptiles is similar to that in birds, but important differences also exist. For example, the time-scale of shell formation is prolonged compared to birds, and many reptiles exhibit simultaneous ovulation in which all eggs in a clutch are released within a short period of time and enter the oviduct one after the other whereas in birds, there is sequential ovulation in which a single mature follicle is ovulated, shelled, and oviposited independently of the other eggs [4].

There is only limited information about the egg proteins of *Crocodylus*. During the analysis of the egg white proteins of five reptilian species—including the Siamese crocodile—seven protein groups were found, including serpine, transferrin precursor/iron binding protein, lysozyme C, teneurin-2 (fragment), interferon-induced GTP-binding protein Mx1, succinate dehydrogenase iron-sulphur subunit and olfactory receptor 46 [5]. In the egg white of the Siamese crocodile, twenty isoforms of transferrin precursor were found [5]. Ovotransferrin, being a major glycoprotein in the reptile egg white, was characterized from *Crocodylus siamensis* [6]. This crocodile ovotransferrin was characterized as a glycoprotein with multiple isoforms with pI from 6.0 to 6.8. Information about reptile egg white proteins is limited and restricted to the identification of a few specific proteins (see e.g. [5]).

The extractable proteins of avian eggshells have been studied extensively and many of them identified. Mann et al. [7] described 520 proteins from an acid-soluble organic matrix of the calcified chicken eggshell layer and some 119 proteins in egg yolk (Mann et al. [8]), 78 proteins in egg white, and 528 proteins in the decalcified eggshell organic matrix, whereas Farinazzo et al. [9] extended the number of yolk proteins to 255. Ahmed et al. [10] merged the results from different extraction/solubilization conditions with various proteomes that enabled the identification of 472, 225, and 488 proteins in the avian eggshell membrane, egg white, and eggshell proteomes, respectively. In-depth proteomic analyses of turkey [11] and quail [12] calcified eggshell proteomes have been published. The number of identified mineralized eggshell proteins of chicken, turkey and quail was 675, 697 and 622, respectively, with an overlap of 311 proteins [13]. The zebra finch eggshell matrix is comprised of 475 accepted protein identifications [13]. Most of these proteins overlapped with those previously mentioned for birds; only 78 identified proteins were new. Some of the proteins were identified as eggshell-specific matrix proteins and were designated ovocleidins and ovocalyxins [14]. The EDTA-insoluble proteinaceous film from the cuticle layer of the chicken eggshell was studied. This film contains three main areas: spots, blotches and the surroundings. In total, 29 proteins were determined (and another eight by less specific "cleavage" with semitrypsin) and their distribution among various areas of the cuticle was found to be inhomogeneous. The most dominant proteins were eggshell-specific ones, ovocleidin-17 and ovocleidin-116 [15].

The presence of a pigment in the eggshell of reptilia has not been proposed before [16]. Of course, as can be expected, pigments are present in a wide range of coloured avian eggshells; however they are also present in "white" eggs. It is thought that these pigments may be synthesised in the uterus and then deposited into the eggshell immediately prior to oviposition [17]. Some results suggest that amount of pigment in the cuticle is lower than that contained within the outer calcareous layer of the shell so maybe pigment is not secreted only in the last hour of oviposition [18]. Pigmentation can play an important role in hatching success, since it can serve as a "camouflage", but may also play a role in gas and temperature exchange, and it is reported that the eggshell thickness and pigmentation mediate variations in development and UV exposure in bird eggs [19]. Eggshell maculation (in the presence of spots) is predominantly due to protoporphyrin, but both biliverdin (antioxidant) and protoporphyrin (pro-oxidant) may be present in eggshells. Because of their role in oxidative stress, the deposition of eggshell pigments may reflect the condition of the female [20].

This research continues our analysis of proteins and pigments from avian eggshell [2, 21–24]. The aim of this work is to describe proteins of the reptile eggshell (specifically Siamese crocodile, *C. siamensis*) and their distribution in different layers of the eggshell. We hypothesized that there will be some similarities, due to similar protective function of the eggshell, as well as differences related to different incubation temperature and environment. These proteins, like in the case of avian eggshells, can play an important role in the antimicrobial defense of the egg. We tried also to determine the presence of the pigments(s), which were hitherto not detected in the reptilian eggshell.

2 Materials and Methods

2.1 Chemicals

Trypsin (TPCK treated, from bovine pancreas, 13,500 units per mg),ammonium bicarbonate and acetonitrile (HPLC-MS grade) protoporphyrin IX, bilivedin and internal standard (5,10,15,20-tetra(4-pyridyl)-21H,23H-porphine).were obtained from Sigma (St. Louis, MO, USA). 2-mercaptoethanol was obtained from Merck (Darmstadt, Germany). All solutions were prepared in MilliQ water (Millipore, Bedford, MA, USA). Empore Octadecyl C18 Extraction disks were purchased from Supelco (Bellefonte, PA, USA).

The eggs were obtained from animals bred in captivity (Crocodile ZOO Protivín (http://www.krokodylizoo.cz), Protivín, Czech Republic). The fertilization status was

ascertained by candling the eggs, and the non-fertilized eggs were clearly identified by the lack of opaque band development across the equator [3]. Since the primary purpose of this work was to obtain crocodile embryos for a different study, each such egg was opened to make sure there was indeed no embryo and to empty the contents.

Upon oviposition into the sand at the animal enclosure, the eggs were collected within 24 h and transferred to a humidified incubator (32 °C). The empty eggs were sampled after 7 days of incubation, by which time it was apparent that no embryonic development has occurred. After opening of the eggs and removal of the contents, the eggshells were rinsed with distilled water and stored at − 80 C until the time of further analysis.

2.2 Sample Preparation

2.2.1 Preparation of Eggshell Fractions

2.2.1.1 EDTA Method The preparation of these fractions utilised a modified version of our previously published method for the analysis of birds' eggshells [2]. Whole eggs (4 eggs together) were washed with water and methanol and two types of sample were prepared:

(i) "Cuticle" fraction. Eggs were treated with 5% (0.13 mol/L) EDTA (pH 7.6) containing 10 mmol/L 2-mercaptoethanol (three times the egg volume) for 60 min at room temperature. The resulting insoluble organic layer left on the egg surface after this partial decalcification was scraped off, collected by washing with water and then centrifuged (1000×*g*, 15 min, room temperature). The resulting pellet was resuspended in water and centrifuged under the above conditions (repeated three times) and then lyophilized.

The supernatant after EDTA treatment was extensively dialyzed against water (3 days at laboratory temperature, adding NaN_3 to prevent microbial contamination) and then lyophilized.

(ii) "Palisade" fraction ("honeycomb layer"). In the next step, the egg that had undergone step A was treated with 0.6 mol/L EDTA (pH 7.6) containing 10 mmol/L 2-mercaptoethanol (three times the egg volume) for 150 min at laboratory temperature. The insoluble material (layer) on the eggs was scraped off and the material was subjected to the same procedure as described in A.

The supernatant after EDTA treatment was extensively dialyzed against water (3 days at laboratory temperature, adding NaN_3 (3 mmol/L) to prevent microbial contamination; (cut off 10,000) and then lyophilized.

2.2.1.2 Acetic Acid Method This method was a slightly modified version of the method used by Mann's group for the analysis of avian eggshell [12] and it is based on discarding the cuticle and mammillary layers and analysing all acid-soluble proteins from the palisade layer.

The eggs (2) were emptied, the shells were cleaned under a jet of water, and washed with 5% EDTA for 60 min at room temperature to facilitate mechanical removal of the cuticle and the membranes. The cuticles were then removed by brushing under a jet of de-ionized water, and pieces of calcified shell were stripped off the wet membranes. The dried pieces of calcified eggshell were demineralized in 50% acetic acid (20 ml/g of shell) at 4–8 °C for 15 h with constant stirring. The turbid mixture was dialyzed (cut off 10,000) with 3 × 10 vol of 10% acetic acid and 3 × 10 vol of 5% acetic acid, and lyophilized.

Trypsin cleavage the samples were incubated at 37 °C in pH 7.8 ammonium bicarbonate buffer (20 mmol/L) added with trypsin (trypsin TPCK treated, from bovine pancreas) (1:50 enzyme:substrate ratio). After 3 h the cleavage was stopped by acidification with acetic acid.

After trypsin cleavage, the samples were purified with StageTips using Empore C18 Extraction disks according to the published protocol [25].

2.2.2 Analysis of Tryptic Digests with LC-MS/MS

The nano-HPLC apparatus used for protein digest analysis was a Proxeon Easy-nLC (Proxeon, Odense, Denmark). It was coupled to an ultrahigh resolution MaXis Q-TOF (quadrupole—time of flight) mass spectrometer (Bruker Daltonics, Bremen, Germany) by nanoelectrosprayer. The nLC-MS/MS instruments were controlled with the software packages HyStar 3.2 and micrOTOF-control 3.0. The data were collected and manipulated with the software packages ProteinScape 3.0 and DataAnalysis 4.0 (Bruker Daltonics).

Five microliters of the peptide mixture were injected into an NS-AC-12dp3-C18 Biosphere C18 column (particle size: 3 µm, pore size: 12 nm, length: 200 mm, inner diameter: 75 µm) with an NS-MP-10 Biosphere C18 precolumn (particle size: 5 µm, pore size: 12 nm, length: 20 mm, inner diameter: 100 µm), both manufactured by NanoSeparations (Nieuwkoop, Holland).

The separation of peptides was achieved via a linear gradient between mobile phase A (water) and B (acetonitrile), both containing 0.1% (v/v) formic acid. Separation was started by running the system with 5% mobile phase B, followed by a gradient elution to 7% B at 5 min, 30% B at 180 min. The next step was a gradient elution to 50% B in 10 min and then a gradient to 100% B in 10 min. Finally, the column was eluted with 100% B for 20 min. Equilibration between the runs was achieved by washing the column with 5% mobile phase B for 10 min. The flow rate was

0.20 µL/min and the column was held at ambient temperature (25 °C).

On-line nano-electrospray ionization (easy nano-ESI) was used in positive mode. The ESI voltage was set to + 4.5 kV, scan time: 3 Hz. Operating conditions: drying gas (N_2): 4 L/min; drying gas temperature: 180 °C; nebulizer pressure: 100 kPa. Experiments were performed by scanning from 50 to 2200 *m/z*. The reference ion used (internal mass lock) was a monocharged ion of $C_{24}H_{19}F_{36}N_3O_6P_3$ (*m/z* 1221.9906). Mass spectra corresponding to each signal from the total ion current chromatogram were averaged, enabling an accurate molecular mass determination. All LC-MS and LC-MS/MS analyses were done in duplicate.

2.2.3 Database Searching

Data were processed using ProteinScape software v. 3.0.0.446 (Bruker Daltonics, Bremen, Germany). Proteins were identified by correlating tandem mass spectra to the extracted database for *Crocodylia* from the NCBI database (downloaded on 10th February 2017; 164,096 proteins), using the MASCOT searching engine v. 2.3.0 (http://www.matrixscience.com). We also tried the same search of the extracted database for *Crocodylia* from the Uniprot database, but we obtained a lower coverage of proteins. Control searching was done on the whole Uniprot database and on the database extracted from NCBI databases for *Aves*. Another search was done in the database IPI chicken v. 3.81 [26] (this database is no longer supported, but the reference to previous papers using it is important). Trypsin was chosen as the enzyme parameter. Three missed cleavages were allowed, and an initial peptide mass tolerance of ± 10.0 ppm was used for MS and ± 0.05 Da for MS/MS analysis. Variable modifications were set: proline and lysine were allowed to be hydroxylated, methionine oxidated, whereas asparagine and glutamine deamidated. In the initial experiments we searched for modifications by using a non-specific search for modifications (errors) to ensure there were no other modifications besides the ones we were looking for. All these possible modifications were set to be variable. The monoisotopic peptide charge was set to 1 +, 2 + and 3 +. The Peptide Decoy option was selected during the data search process to remove false-positive results. Only significant hits were accepted (MASCOT score ≥ 80 for proteins and MASCOT score ≥ 20 for peptides, http://www.matrixscience.com), however all peptides and proteins were additionally manually validated.

The relative abundances of identified proteins were estimated quantitatively by calculating their exponentially modified protein abundance index (emPAI) which is 10^{PAI-1} [27, 28], where PAI is the number of observed peptides divided by the number of theoretically observable peptides. For emPAI calculations, the following criteria were applied:

1. Peptide sequences that were present in several different forms (peptides bearing different charges or those that were modified) were considered as one peptide for the purpose of calculating the number of observed peptides, providing that the peptide had Mr in the range 600–6000.
2. For peptides containing missed cleavage(s), only those partial peptides with Mr in the range 600–6000 were used for calculating the number of observed peptides, providing that at the same time they weren't present in tryptic digests with zero missed cleavages
3. The number of theoretically observable peptides was determined using a software tool available online (http://web.expasy.org/peptide_mass/); the number of missed cleavages was set to zero; only peptides with Mr in the range 600–6000 were used for the calculation; when a protein contained duplicate/repeating peptide sequences, such sequences were considered as one peptide for the purpose of calculating the number of theoretically observable peptides

2.2.4 Pigment analysis

For pigment analyses we used 21 egg shells (weight 8–14 g). After collection, these eggs were stored in a freezer (-20 °C).

Protoporphyrin IX and biliverdin were quantified in the form of their dimethylesters (following Miksik et al. [23, 29]). Pigments were extracted and esterified in absolute methanol (15 ml) containing concentrated sulphuric acid (5%) at room temperature in the dark under N_2 for 24 h. Extract solutions were decanted and chloroform (10 ml) and distilled water (10 ml) were added, then the mix was shaken. The lower (chloroform) phase was collected, and the upper (aqueous) phase extracted with chloroform again (chloroform phases from both extractions were collected). These phases were washed in 10% NaCl (5 ml), followed by distilled water until the wash solution was neutral. Extracts were evaporated to dryness and reconstituted in chloroform (0.25 ml) with an internal standard (5,10,15,20-tetra(4-pyridyl)-21H,23H-porphine; 0.01 mg/ml). Commercially sourced standards for quantification (protoporphyrin IX and biliverdin) were treated using the same procedure.

Pigments were identified and their concentration quantified by reversed-phase high-performance chromatography using a gradient elution between water and acetonitrile with formic acid, and compounds were monitored by UV absorbance, fluorescence and with an ion-trap mass spectrometer (using multiple reaction monitoring).

3 Results

Proteins were analyzed from two parts of the crocodile's eggshell—the outermost and the main part, the cuticle and palisade layer respectively, which resembles the situation in the eggshell of birds. However, as was mentioned in the Introduction, the eggshell of *Alligator mississippiensis* can be divided into five layers: the outermost layer is a densely calcified layer (100–200 µm thick), then comes a honeycomb layer (300–400 µm thick), an organic layer (8–12 µm thick), a mammillary layer (20–29 µm thick), and the shell membrane (150–250 µm thick) [3]. So the first layer looks like cuticle layer to the eggshell of birds and the honeycomb layer resembles the palisade layer. The mammillary layer and shell membrane exist also in the eggshell of birds. We assume that the outermost part (cuticle) is responsible for protection against the external environment and the palisade part for the mechanical strength of the eggshell.

We identified 58 proteins in the whole eggshell matrix, of which 5 were specific for the cuticle and 26 for the palisade (see Table 1). The cuticle contained a lower number of proteins (32) and the relative distribution of proteins was slightly different in both layers.

In an attempt to increase the number of discovered proteins we tried to use PAGE analysis (when we cut-off PAGE strip to 10 bands for analysis by nLC-MS), but without success, i.e. without any new protein being identified (data not shown).

The dominant proteins were thyroglobulin, mucin-5AC, lysyl oxidase (in the pallisade layer), IgGFc-binding protein, ovostatin, calumenin, vitellogenins (in the cuticle layer) and apolipoprotein B-100 (in the cuticle layer)—see Table 2 (for individually identified peptides of these proteins see Supplementary Table).

The most abundant proteins in the eggshell of crocodile (according to emPAI as well as Mascot score) are thyroglobulin, IgGFc-binding protein-like, calumenin and lysyl oxidase-like 2.

Besides searching for proteins using trypsin cleavage we also used semitrypsine approach. In this case we found two additional proteins: XP_019398538.1—PREDICTED: galactose-3-*O*-sulfotransferase 2 [*Crocodylus porosus*] (Mascot Score = 250) and XP_019393650.1—PREDICTED: cathepsin B [*Crocodylus porosus*] (Mascot score = 121).

Table 1 Number of proteins in eggshell of *Crocodylus siamensis*

	Proteins	
	Total	Specific
Overall	58	
Palisade	54	26
Cuticle	32	4

The roles of the described proteins were analyzed using the Panther classification system (http://pantherdb.org/) in which searches were made for corresponding human proteins (a dataset for crocodiles does not exist)—see Fig. 1. It is evident that the majority of the proteins have a binding function and they participate in the cellular process. There are also defense/immunity proteins and enzyme modulators present. Calcium binding protein is present as well.

We also identified a pigment, protoporhyrin IX, in the crocodile eggshell (despite the fact that it was previously described that reptile eggshells don not contain any [16])—but at a really low concentration: 0.90 ± 0.73 ng/g, i.e. 1.61 ± 1.30 pmol/g ($n = 21$; mean ± SD). We did the measurement on the extract from the entire eggshell only. To exclude possible contamination we peeled away pieces of eggshell(s) from intact inner membrane of the egg. The eggshells were rinsed extensively with distilled water (3-times).

4 Discussion

Differences in the proportions of proteins between the two layers (cuticle and palisade—see Table 1) were also reported for the chicken eggshell [2]; however in that case the particular proteins are different.

The relative protein composition of the crocodile eggshell differs from the avian eggshell (see Table 2). Among the proteins reported in the avian shell proteome, we found 41 similar proteins (33 in chicken, 30 in quail, 32 in turkey and 27 in zebra finch shells, and 23 proteins are reported for all four bird species) [7, 11–13]. Although this number may appear high (70% of proteins were reported in crocodilian as well as avian eggshells), it is noteworthy that the proteins are similar (belong to the same group of proteins), but not identical. As we stated, the relative proportions are totally different. For example, in the chicken eggshells, the highly abundant proteins (determined by emPAI values) are eggshell-specific proteins like ovocleidin-17, ovocleidin-116, ovocalyxin-32, and ovocalyxin-36, as well as clusterin [7]. Of these avian eggshell-specific proteins, only clusterin was detected in the eggshells of the Siamese crocodile, but it was not as abundant and only found in the palisade layer (32nd according to Mascot score).

The most abundant proteins in the eggshell of crocodile (according to emPAI as well as Mascot score) are thyroglobulin, IgGFc-binding protein-like, calumenin and lysyl oxidase-like 2. Some similar proteins can be detected in the avian eggshell, however in significantly lower abundance. The discovered transferrin precursors were previously reported as major components in reptile egg white (when five reptile species were studied, namely Siamese crocodile (*Crocodylus siamensis*), soft-shelled turtle (*Trionyx sinensis taiwanese*), red-eared slider turtle (*Trachemys scripta*

Table 2 Merged results for proteins of overall eggshell, palisade (main layer) and cuticle (surface layer) eggshell matrix proteins of *Crocodylus siamensis*

Rank	Accession	Protein	MW (kDa)	pI	Summary			Palisade			Cuticle			Similar protein present in avian shell proteome
					Mascot score	Sequence coverage (%)	emPAI	Mascot score	Sequence coverage (%)	emPAI	Mascot score	Sequence coverage (%)	emPAI	
1	XP_019403055.1	PREDICTED: thyroglobulin [*Crocodylus porosus*]	304.4	5.0	10128	38.9	2.48	6408	29.0	1.61	9320	37.3	2.32	q
	XP 019375187.1	PREDICTED: thyroglobulin [*Gavialis gangeticus*]	304.3	5.0	7192	25.9	1.42	4488	19.3	0.97	6745	25.3	1.34	
2	XP_019406548.1	PREDICTED: mucin-5AC [*Crocodylus porosus*]	269.3	5.7	2468	14.3	0.71	1909	12.2	0.60	1986	11.5	0.57	c, q, t, z
	XP_019375532.1	PREDICTED: mucin-5AC [*Gavialis gangeticus*]	279.1	5.4	1301	7.6	0.36	845	5.9	0.25	952	6.4	0.29	
	XP 019406547.1	PREDICTED: mucin-5AC-like [*Crocodylus porosus*]	31.5	5.9	522	24.8	1.05	230	12.9	0.54	412	19.4	0.78	
3	XP 019412127.1	PREDICTED: lysyl oxidase homolog 2 [*Crocodylus porosus*]	86.8	6.3	2507	27.2	1.70	2507	27.2	1.70	–	–	–	c, q, t, z
4	XP_014381440.1	PREDICTED: IgGFc-binding protein-like [*Alligator sinensis*]	538.5	10.1	2311	8.7	0.47	2283	8.6	0.45	269	1.0	0.05	c, q, t, z
	XP 019355496.1	PREDICTED: IgGFc-binding protein [*Alligator mississippiensis*]	315.3	10.5	2034	12.1	0.60	2034	12.1	0.60	–	–	–	

Table 2 (continued)

Rank	Accession	Protein	MW (kDa)	pI	Summary			Palisade			Cuticle			Similar protein present in avian shell proteome
					Mascot score	Sequence coverage (%)	emPAI	Mascot score	Sequence coverage (%)	emPAI	Mascot score	Sequence coverage (%)	emPAI	
5	XP_019399677.1	PREDICTED: ovostatin-like [*Crocodylus porosus*]	151.8	7.6	2295	28.2	1.35	2269	28.2	1.35	928	14.0	0.55	c, q, t, z
	XP 019371617.1	PREDICTED: ovostatin-like [*Gavialis gangeticus*]	162.9	6.8	1833	21.8	1.00	1779	21.8	1.00	763	10.4	0.43	
6	KYO31913.1	Calumenin isoform D [*Alligator mississippiensis*]	45.6	4.3	1881	63.5	5.06	1457	57.0	4.48	1468	57.3	3.96	c, z
7	XP 019386149.1	PREDICTED: vitellogenin-2-like [*Crocodylus porosus*]	206.0	9.6	1665	19.2	0.91	49	0.5	0.02	1652	19.2	0.91	c, q, t, z
8	XP 019386152.1	PREDICTED: vitellogenin-1-like [*Crocodylus porosus*]	205.8	9.8	1475	16.9	0.69	101	1.3	0.04	1456	16.9	0.69	c, q, t, z
9	XP_019395043.1	PREDICTED: uncharacterized protein LOC109313096 [*Crocodylus porosus*]	53.3	10.3	1184	33.3	1.71	1184	33.3	1.71	684	23.0	1.00	
10	XP 019392884.1	PREDICTED: apolipoprotein B-100 [*Crocodylus porosus*]	522.8	8.6	949	4.8	0.15	–	–	–	949	4.8	0.15	c, z
11	XP 019395327.1	PREDICTED: protein TENP-like [*Crocodylus porosus*]	44.4	9.4	934	23.7	1.23	355	16.7	0.82	919	23.7	1.23	c, t, z

Table 2 (continued)

Rank	Accession	Protein	MW (kDa)	pI	Summary			Palisade			Cuticle			Similar protein present in avian shell proteome
					Mascot score	Sequence coverage (%)	emPAI	Mascot score	Sequence coverage (%)	emPAI	Mascot score	Sequence coverage (%)	emPAI	
12	XP_019407814.1	PREDICTED: ectonucleotide pyrophosphatase/phosphodiesterase family member 2 isoform X3 [*Crocodylus porosus*]	98.4	8.3	930	14.5	0.53	716	13.2	0.46	865	14.3	0.53	c, t
13	XP 019406546.1	PREDICTED: mucin-5B-like [*Crocodylus porosus*]	235.5	5.5	809	4.3	0.15	809	4.3	0.15	–	–	–	c, q, t, z
14	XP 006029690.1	PREDICTED: serum amyloid P-component-like [*Alligator sinensis*]	27.0	6.1	542	27.0	1.68	542	27.0	1.68	89	6.3	0.39	
15	KYO40443.1	Complement C5 [*Alligator mississippiensis*]	176.1	6.4	529	6.8	0.23	383	4.7	0.17	344	4.9	0.15	
16	XP_019390939.1	PREDICTED: serine protease inhibitor Kazal-type 1-like [*Crocodylus porosus*]	9.4	9.1	513	45.9	2.98	436	45.9	2.98	470	45.9	2.98	t
17	XP 019404016.1	PREDICTED: angiotensin-converting enzyme [*Crocodylus porosus*]	147.8	6.1	497	8.1	0.25	497	8.1	0.25	–	–	–	
18	XP 014380747.1	PREDICTED: actin, cytoplasmic 2 isoform X2 [*Alligator sinensis*]	41.7	5.3	442	20.8	1.02	442	20.8	1.02	–	–	–	c, q

Table 2 (continued)

Rank	Accession	Protein	MW (kDa)	pI	Summary			Palisade			Cuticle			Similar protein present in avian shell proteome
					Mascot score	Sequence coverage (%)	emPAI	Mascot score	Sequence coverage (%)	emPAI	Mascot score	Sequence coverage (%)	emPAI	
19	XP_019389284.1	PREDICTED: hyaluronan and proteoglycan link protein 3 [*Crocodylus porosus*]	41.0	9.4	418	23.8	0.93	370	19.9	0.78	95	5.8	0.18	c, q, t, z
	KYO21076.1	EGF-like repeat and discoidin I-like domain-containing protein 3 [*Alligator mississippiensis*]	94.8	9.5	308	7.7	0.25	308	7.7	0.25	–	–	–	
20	XP_019402426.1	PREDICTED: polymeric immunoglobulin receptor [*Crocodylus porosus*]	69.2	5.3	372	10.3	0.39	367	10.3	0.39	207	5.1	0.22	c, q, t
21	XP_019357858.1	PREDICTED: pulmonary surfactant-associated protein D-like [*Gavialis gangeticus*]	27.1	5.6	364	16.4	0.50	364	16.4	0.50	–	–	–	
22	CAK18230.1	Transferrin precursor [*Crocodylus niloticus*]	76.2	6.4	294	9.4	0.28	294	9.4	0.28	97	3.9	0.11	
23	XP 019399510.1	PREDICTED: pendrin [*Crocodylus porosus*]	82.7	9.5	276	8.1	0.29	276	8.1	0.29	249	8.1	0.29	
24	XP 019406544.1	PREDICTED: mucin-6 [*Crocodylus porosus*]	136.3	5.9	274	3.5	0.13	274	3.5	0.13	–	–	–	
25	XP 019407475.1	PREDICTED: CD44 antigen [*Crocodylus porosus*]	123.7	4.9	265	3.3	0.17	265	3.3	0.17	–	–	–	

Table 2 (continued)

Rank	Accession	Protein	MW (kDa)	pI	Summary			Palisade			Cuticle			Similar protein present in avian shell proteome
					Mascot score	Sequence coverage (%)	emPAI	Mascot score	Sequence coverage (%)	emPAI	Mascot score	Sequence coverage (%)	emPAI	
26	XP 006023890.1	PREDICTED: annexin A1 [*Alligator sinensis*]	38.5	6.4	231	16.7	0.47	231	16.7	0.47	61	3.5	0.10	c, q, t, z
27	XP_019399690.1	PREDICTED: alpha-2-macroglobulin-like protein 1 isoform X3 [*Crocodylus porosus*]	145.5	5.1	191	3.3	0.15	170	3.3	0.15	135	2.3	0.11	c, q. t, z
28	AFZ39220.1	Secreted immunoglobulin upsilon3 heavy chain, partial [*Crocodylus siamensis*]	47.2	9.8	190	7.9	0.35	190	7.9	0.35	46	2.6	0.11	
29	KYO46236.1	Carboxypeptidase E [*Alligator mississippiensis*]	45.5	5.0	188	8.4	0.37	124	5.2	0.17	138	6.5	0.27	c, q, z
30	XP_019359922.1	PREDICTED: carbonic anhydrase 12 isoform X3 [*Gavialis gangeticus*]	42.2	7.9	183	7.9	0.25	173	7.9	0.25	183	7.9	0.25	c, q, t, z
31	XP 019400724.1	PREDICTED: matrix Gla protein [*Crocodylus porosus*]	12.4	10.0	177	25.2	1.51	177	25.2	1.51	81	20.4	1.51	
32	XP 006274713.1	PREDICTED: clusterin [*Alligator mississippiensis*]	53.3	5.0	168	9.9	0.25	168	9.9	0.25	–	–	–	c, q, t
33	XP_019400352.1	PREDICTED: BPI fold-containing family C protein-like [*Crocodylus porosus*]	53.3	9.8	166	10.5	0.41	166	10.5	0.41	–	–	–	q

Table 2 (continued)

Rank	Accession	Protein	MW (kDa)	pI	Summary			Palisade			Cuticle			Similar protein present in avian shell proteome
					Mascot score	Sequence coverage (%)	emPAI	Mascot score	Sequence coverage (%)	emPAI	Mascot score	Sequence coverage (%)	emPAI	
34	XP_014375690.1	PREDICTED: uncharacterized protein LOC102387935 [*Alligator sinensis*]	81.2	11.1	162	4.4	0.18	162	4.4	0.18	–	–	–	
35	XP_019393182.1	PREDICTED: beta-galactosidase-like isoform X1 [*Crocodylus porosus*]	73.2	5.1	159	5.5	0.20	158	5.5	0.20	101	2.8	0.06	c, q, t, z
36	XP 019384928.1	PREDICTED: CD99 antigen-like isoform X5 [*Crocodylus porosus*]	17.5	5.6	149	15.6	0.67	148	15.6	0.67	77	10.8	0.29	
37	BAN62838.1	Hemoglobin subunit alpha [*Crocodylus siamensis*]	15.7	8.0	142	21.8	0.58	142	21.8	0.58	–	–	–	c, q, t, z
38	XP_019351818.1	PREDICTED: phospholipase A2 inhibitor and Ly6/PLAUR domain-containing protein-like isoform X1 [*Alligator mississippiensis*]	28.6	10.9	130	5.2	0.15	130	5.2	0.15	–	–	–	
39	XP 019359193.1	PREDICTED: glia-derived nexin isoform X2 [*Gavialis gangeticus*]	44.0	10.1	124	6.3	0.21	124	6.3	0.21	–	–	–	c, q, t, z

Table 2 (continued)

Rank	Accession	Protein	MW (kDa)	pI	Summary			Palisade			Cuticle			Similar protein present in avian shell proteome
					Mascot score	Sequence coverage (%)	emPAI	Mascot score	Sequence coverage (%)	emPAI	Mascot score	Sequence coverage (%)	emPAI	
40	XP_019371610.1	PREDICTED: alpha-2-macroglobulin-like protein 1 isoform X2 [*Gavialis gangeticus*]	143.6	9.1	120	2.1	0.09	–	–	–	120	2.1	0.09	c, q, t, z
41	KYO18102.1	Alpha-1,6-mannosylglycoprotein 6-beta-*N*-acetylglucosaminyltransferase A [*Alligator mississippiensis*]	81.6	9.2	120	1.7	0.05	120	1.7	0.05	–	–	–	c, q, t, z
42	XP_019409946.1	PREDICTED: polypeptide *N*-acetylgalactosaminyltransferase 5 [*Crocodylus porosus*]	109.0	10.1	117	2.7	0.07	117	2.7	0.07	–	–	–	c, q, t, z
43	XP 006018871.1	PREDICTED: 45 kDa calcium-binding protein [*Alligator sinensis*]	41.5	4.6	115	7.9	0.15	115	7.9	0.15	–	–	–	c, q, t, z
44	XP_014373761.1	PREDICTED: limbic system-associated membrane protein [*Alligator sinensis*]	34.8	5.1	115	5.1	0.12	115	5.1	0.12	–	–	–	z
45	XP_006260729.2	PREDICTED: leucine-rich repeat-containing G-protein coupled receptor 4 isoform X1 [*Alligator mississippiensis*]	104.9	5.8	107	2.0	0.11	107	2.0	0.11	53	0.9	0.05	

Table 2 (continued)

Rank	Accession	Protein	MW (kDa)	pI	Summary			Palisade			Cuticle			Similar protein present in avian shell proteome
					Mascot score	Sequence coverage (%)	emPAI	Mascot score	Sequence coverage (%)	emPAI	Mascot score	Sequence coverage (%)	emPAI	
46	XP 014452266.1	PREDICTED: ezrin [*Alligator mississippiensis*]	69.5	5.7	106	4.4	0.11	106	4.4	0.11	57	1.5	0.05	c, q, t, z
47	XP_019366071.1	PREDICTED: alpha-1-acid glycoprotein 1-like isoform X2 [*Gavialis gangeticus*]	22.4	4.8	106	12.4	0.36	106	12.4	0.36	–	–	–	
48	XP 019404349.1	PREDICTED: apovitellenin-1-like [*Crocodylus porosus*]	11.9	9.8	104	17.1	0.93	–	–	–	104	17.1	0.93	c, q, t, z
49	KYO26756.1	Anterior gradient protein 3 [*Alligator mississippiensis*]	16.9	6.1	98	15.2	0.67	98	15.2	0.67	–	–	–	t
50	XP 019402744.1	PREDICTED: plasma protease C1 inhibitor [*Crocodylus porosus*]	61.0	4.7	95	4.0	0.08	95	4.0	0.08	–	–	–	c, q, t, z
51	KYO46124.1	Ectonucleotide pyrophosphatase/phosphodiesterase family member 6 [*Alligator mississippiensis*]	48.3	6.5	95	5.0	0.16	95	5.0	0.16	–	–	–	c, t
52	XP 006035570.1	PREDICTED: mucin-16 [*Alligator sinensis*]	142.0	9.8	93	1.6	0.06	93	1.6	0.06	–	–	–	c
53	XP 019389661.1	PREDICTED: complement factor I isoform X7 [*Crocodylus porosus*]	69.2	9.3	86	4.7	0.11	–	–	–	86	4.7	0.11	

Table 2 (continued)

Rank	Accession	Protein	MW (kDa)	pI	Summary			Palisade			Cuticle			Similar protein present in avian shell proteome
					Mascot score	Sequence coverage (%)	emPAI	Mascot score	Sequence coverage (%)	emPAI	Mascot score	Sequence coverage (%)	emPAI	
54	XP 019401834.1	PREDICTED: sortilin isoform X1 [*Crocodylus porosus*]	85.7	5.8	85	2.7	0.11	85	2.7	0.11	–	–	–	t
55	KYO27809.1	Tubulin beta-6 chain [*Alligator mississippiensis*]	38.6	4.6	85	5.2	0.13	85	5.2	0.13	–	–	–	c, q, t
56	XP_019337099.1	PREDICTED: creatine kinase B-type isoform X2 [*Alligator mississippiensis*]	41.4	5.4	82	4.6	0.10	82	4.6	0.10	–	–	–	c, q, t, z
57	XP_019384031.1	PREDICTED: receptor-type tyrosine-protein phosphatase N2 [*Crocodylus porosus*]	116.3	5.4	80	2.8	0.03	80	2.8	0.03	–	–	–	c, q, t
58	AFZ39211.1	Secreted immunoglobulin alpha1 heavy chain, partial [*Crocodylus siamensis*]	49.4	6.9	66	2.2	0.10	66	2.2	0.10	65	2.2	0.10	

The proteins were sorted according mascot score (Score). *SC* sequence coverage, *emPAI* relative abundances using the exponentially modified protein. Similar proteins present in avian shell proteome—identified proteins from published proteomes of chicken (c), quail (q), turkey (t) and zebra finch (z) [7, 11–13]

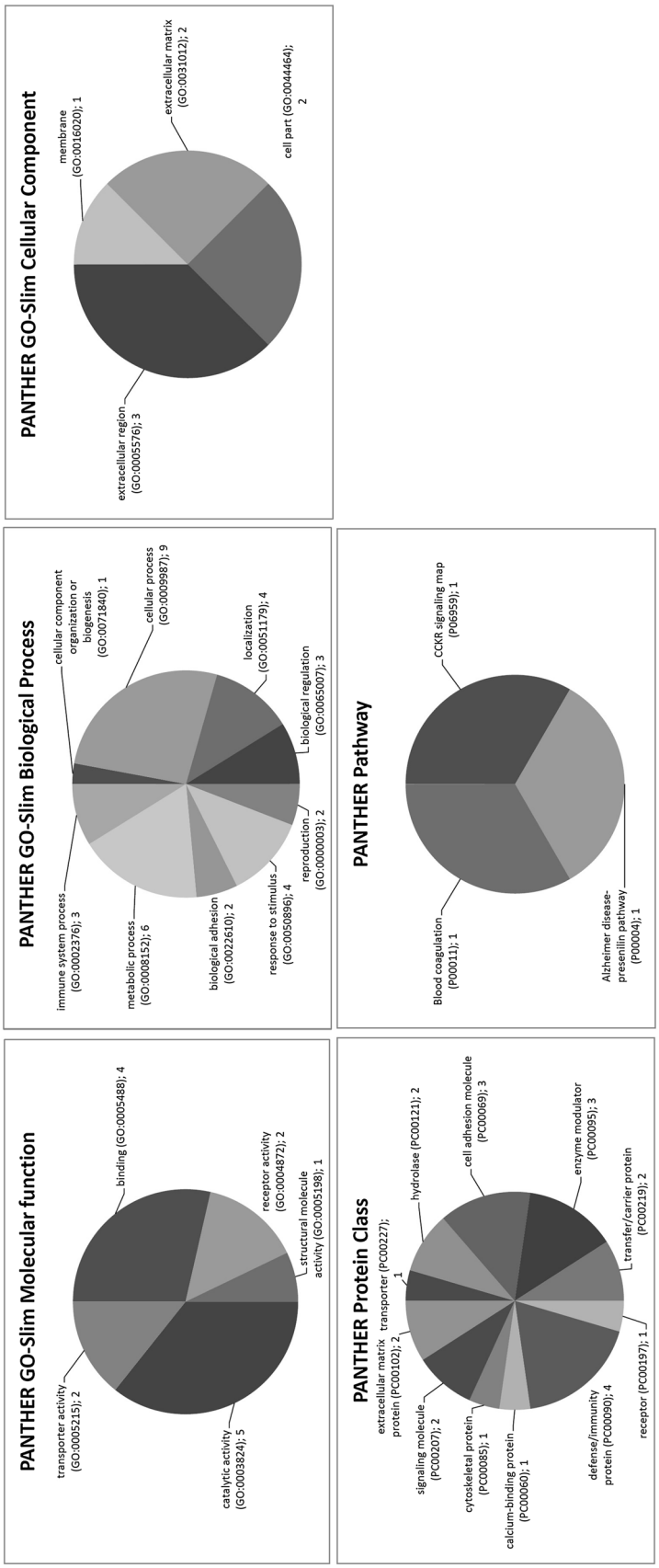

Fig. 1 Protein classification according to Panther (Protein ANalysis Through Evolutionary Relationships) classification system (http://pantherdb.org/) - searches were made for human corresponding proteins

elegans), hawksbill turtle (*Eretmochelys imbricate*) and green turtle (*Chelonia mydas*)) [5].

Thyroglobulin is produced by the follicular cells of the thyroid and used entirely within the thyroid gland. The thyroid also produces the hormone calcitonin, which plays a role in calcium homeostasis. Regretably this hormone was not discovered in the eggshell although it was present in searched database (assigned to *Alligator mississippiensis* and *Alligator sinensis*). Calcitonin was reported in the eggshell of zebrafinch (calcitonin gene-related peptide 2 (CALCB)/procalcitonin) only [13]. Thyroglobulin was previously only determined in the proteome of quail eggshell, but in a low abundance.

We can speculate whether there are also some "reptile eggshell-specific" proteins; however, the full proteome of these animals is not yet available. Proteome of the Siamese crocodile eggshell is insufficiently described (e.g. look at the Table 2, where only 3 proteins are ascribed to *Crocodylus siamensis*). Species differences in sequence of orthologs can lead to low identification success. We have to mention that in the Crocodylia database are present two proteins similar to proteins that play an important role in avian eggshell mineralization: ovocleidin-116 (precursor) for *Alligator mississippiensis* as well as clusterin (*Alligator mississippiensis, Alligator sinensis, Crocodylus porosus*) and osteopontin (*A. mississippiensis, A. sinensis, C. porosus, Gavialis gangeticus*), and these proteins were not detected in the present study. In our search in the proteomic database we did not find any connections (after searching the database allowing for some "errors" in the sequence) to avian eggshell-specific proteins. These specific proteins probably play an important role in the antimicrobial defense of the eggs (besides other proteins, like Kunitz-like protease inhibitor), mainly in the cuticle (outermost layer) [21, 30]. However, we detected enzymes (e.g. lysyl oxidase, ectonucleotide pyrophosphatase/phosphodiesterase, carboxypetidase E), and mainly serine protease inhibitor Kazal-type 1, which can serve as a Kunitz-like protease inhibitor in avian eggshell. This protease inhibitor is relatively abundant (emPAI = 2.98, when Kunitz-like protease inhibitor in the cuticle of chicken eggshell had emPAI = 1.04 [15]). Serine protease inhibitor Kazal-type 1 is described as a pancreatic secretory trypsin-inhibitor and is strongly elevated in pancreatitis, in which its role is not just as a trypsin inhibitor, but also as a growth factor as well as a negative regulator of autophagy [31].

Enzyme lysyl oxidase-2 can crosslink collagen (which was described in the chicken eggshell [2]) however no collagen was found in the present study (using searching of *Crocodylia* as well *Aves* data set).

We determined the presence of the pigment, protoporhyrin IX, in the crocodile eggshell for the first time; it was previously described that reptile eggshells do not contain any [16]—but at a really low concentration: 0.90 ± 0.73 ng/g, i.e. 1.61 ± 1.30 pmol/g (n = 21; mean ± SD). If we compare these data with published ones, the concentration is about 2–3 orders of magnitude lower than in the white avian eggs—Swift (*Apus apus*) 2.90 nmol/g [32] or the greenish eggs of the Spotless starling (*Sturnus unicolor*) 0.18 ± 0.10 (n = 80) nmol/g [33]. For example, the range of protoporhyrin content for British breeding non-passerine birds was from 0.36 nmol/g (*Fulmarus glacialis*) to 478 nmol/g (*Vanellus vanellus*) [29].

However, in the birds' eggs, two pigments were detected—protoporphyrin IX and biliverdin. Whereas protoporphyrin IX results in a yellow–brown coloration, biliverdin is responsible for greenish coloration. In principle, biliverdin is present at a significantly lower level than proporphyrin IX (depending on the individual bird species, but on average it is ten times lower). In our measurements, the limit of detection of biliverdin was 0.5 pmol/g of eggshell, so it is not surprising that biliverdin was not detected in the crocodile eggs. It has to be noted that, for any measurement of compounds in biological samples, the question of sensitivity is of paramount importance. So "zero" does not necessary mean the absence of a compound in the sample, it merely means that it is below the limit of detection. For this reason, we can only speculate whether biliverdin is also present in the eggshell of crocodile at a lower concentration or not at all. In birds, it is proposed that eggshell protoporphyrin and biliverdin concentrations are positively correlated, and it is thought that these pigments are most likely derived from the same precursor metabolic pathway [24, 34]. It was found that those concentrations positively correlate across species, and also exhibit strongly co-varying phylogenetic patterns [29]. No single hypothesis is likely to explain the diversity in eggshell coloration and patterning across birds, suggesting that eggshell appearance is most likely to have evolved to fulfill many nonexclusive functions [24]. Birds' eggshell pigment concentrations, and their resultant coloration of eggs laid by passerines, are largely explained by the evolutionary history of species in a multispecies comparison, and only to a lesser extent by nesting ecology and life-history traits [24, 29].

5 Conclusion

This is a first attempt to describe the proteome of a reptile eggshell. In comparison to the proteins determined in avian eggshell, we observed substantial differences in quality (41 similar proteins, when avian eggshell-specific proteins are missing) as well as quantity of proteins, however the entire proteome of *Crocodilians* is not yet available.

Protein composition is different in the surface (cuticle) and honeycomb (palisade) layers. These layers could be important for antimicrobial defense of egg (mainly cuticle).

The presence of a porphyrin pigment in a crocodilian eggshell is reported for the first time.

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Compliance with Ethical Standards

Conflict of interest All authors declare that there are no competing interests.

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