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Ninhydrin staining and SDS PAGE analysis revealed variations in egg shell surface and water soluble egg shell proteins in a few Avian eggs

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Abstract
The present work was aimed at analyzing the protein of egg shell and its membrane involving three methodology yielded a confirmative results. The ninhydrin stained egg shell surface had shown positive rheumann purple coloration in three species *Gallus gallus domesticus* (Poultry chicken), *Gallus gallus* (Native chicken) and Griraja chicken but not in *Columbia livia* (pigeon). The shell pores were clearly visible in three egg shell species of family Galidae, but in *Columbia livia* pores appeared as dented depression on the shell. The qualitative estimation of shell protein showed variation in the quantity. Least Quantity of 0.02mg/100 mg in Gallus*gallus domesticus* and highest quantity of protein 0.25mg/100 mg in *Columbia livia* was recorded. The electrophoretic analysis of water soluble protein manifested different in all the four species, of these highest molecular weight of 63.09 KDa and 44.04KDa recorded in *columbialivia* that had two types of protein. In Giriraja and *Gallus gallus* species single band of protein having molecular weight 39.21KDa and 44.05KDa represented respectively. In *Gallus gallus domesticus* two bands of lower molecular weight of 21.98 KDa and 8.91 KDa were recorded. This study has confirmed the importance of Ninhydin staining in the study of egg shell profile as well deciphered the existence of water soluble protein in egg shell.

Keywords: Shell pores, eggshell protein, ninhydrinstaining, electrophoresis

1. Introduction
Avian egg shell is highly organized and mineralized structure essential for protection of the growing embryo and propagation of bird species [1]. The egg shell protects the content and embryo from microbes and physical environment. The avian egg shell consists of three layers the most internal are shell membranes which contain collagens as major component the intermediate calcified zone and outer most cuticle. The shell constitute about 3.3% protein of the total hen's egg based on wet weight [2]. Antibacterial proteins like lysozyme and ova transferrin are also present in the egg shell because of their role during egg shell formation and their anti-bacterial property, egg shell proteins are thought to be involved in natural egg defenses by reinforcing the mineral structure and by expressing anti-bacterial activity which preserve the hygienic quality of eggs during its formation [3]. Earlier a team of workers along with the senior authors had shown that the shell surface protein could be visualized by staining with either Giemsa or Ninhydrin that added new initiation for egg shell surface study by simple technique [4, 5]. These proteins are produced by cells of the shell gland mucosa, tends to form larger aggregates [6]. The other aspects of avian shell have involved studies on colour of the shell speckles, maculations and pores. Substantial work has been carried by different researchers to understand the structure and function of shell pores. Our analysis of egg shell surface proteins, pores and water soluble proteins in the shell has been discussed. The objective of this study was to know about integrated proteins of the egg shell and ninhydrin positive protein analysis on the egg shell surface of a few avian eggshells has yielded a convincing results.

2. Materials and Methods
This research work was carried during February-2017 to May 2017.The egg of Poultry chicken (*Gallus gallus domesticus*), Native chicken (*gallus gallus*), Giriraja chicken and pigeon (*Columbia livia*) collected in and around Mysuru taluk in Karnataka. Freshly broken empty egg shell outer surface stained using ninhydrin as per the procedure described by Dinesh Udapi et al [4] and are photographed.

2.1 Ninhydrin staining
About 10 ml of 2% ninhydrin solution was taken in a petridish, warmed and the outer surface of the egg shell was dipped in the ninhydrin solution and allowed to dry for the observation of dots and pores.

2.2 Photography
The ninhydrin treated shells were photographed using mobile phone camera (13pixel) attached with a macro lens, exposed keeping against the naturallight source, obtained superior picture.

2.3 Protein analysis by SDS – PAGE method.
To estimate the water soluble proteins in the different egg shells, SDS-PAGE method is employed [7]. The egg shell of different birds rinsed with water and ground to a powder with pestle and mortar by adding 10ml of water that had laboratory temperature of about 23 ^{0}C. The extraction centrifuged at 12000 rpm for 10 minutes and the resulting supernatant collected used as sample for further analysis.

2.4 Quantitative estimation of protein:
The quantitative estimation of protein in the egg shell of each species was done by following the method described by Lowry et al [8] and the amount of protein in each sample was calculated by graphical method.

2.5 Statistical analysis
Quantity of Protein in egg shell estimated by graphical method. (Graph.1) Series of protein standard ranging from 0-100 µg per ml prepared and optical density is read to construct standard graphical curve. On this standard line optical density of egg shell samples are marked and extrapolated towards X-axis to obtain the concentration of protein. The graph depicts concentration of protein on the X-axis and optical density on the Y-axis.
Quality of protein is estimated by comparing the protein in the eggshell with that of marker. Marker contains known molecular weight proteins. For the molecular weight of these protein log is calculated and marked on the y-axis.
The retardation factor is marked on x-axis. Retardation factor is calculated by using the formula

$$\text{Retardation factor} = \frac{\text{Distance the protein travel on the gel}}{\text{Distance the solvent travel on the gel}}$$

The graph is plotted using the Log (MW) and Rf value of marker to obtain slope (m) in the equation y = mx + c, whereas y = y axis, x = x axis, m = slope and c = intercept.
A standard line is obtained on the Graph. On the line retardation factor (x2) and negative of this retardation factor of proteins in the eggshells is marked (x1). These two points are extrapolated towards the standard line and coinciding points are x1 and y1 respectively. Now slope (m) is calculated by using formula. $m = (y2 - y1)/(x2 - x1)$ (Graph 1).The inverse of log 10^y is the molecular weight of proteins identified in the eggshells of different birds.

3. Results
The egg shell of four different types namely *Gallus gallus domesticus*, *Gallus gallus*, Giriraja and *Columbia livia* stained with Ninhydrin and tested for presence of egg shell surface protein revealed numerous dotted structure positively stained bearing color characteristics to Ninhydrin reaction in all the members of the family Galidae but the shell membrane underlying the shell pores did not show positive reaction with Ninhydrin indicating the absence of ninhydrin positive proteins on the shell membranes. The shell surface of Pigeon egg did not reveal such similar dotted structures on its surface (Fig-1b).The Ninhydrin stained egg shell surface of poultry chicken revealed cluster of protein and also two types of pores small and large scattered on surface viewed with brightly illuminated background (Fig-1a). Compared to the standard poultry chicken egg shell the native chicken egg shell revealed presence of small pores and dots of variable size as scattered in different shape (Fig-1c). Where as in pigeon egg shell there are minute pores and dots which were least visible on surface and its shell surface on reacting with Ninhydrin had continuous stretch of faint rheuman colour unlike dotted appearance in other egg types (Fig -2b). The Giriraja egg shell had two different region on its surface one end was with markings or speckles and the other end was clear (Fig-2a) this bird also produce an egg type without any kind of markings as plain as that of native fowl but both type of eggs almost had similar shell surface profile (Fig-2d). The clear surface on staining with Ninhydrin revealed scattered protein also showed small pores and large dots with depressions along with small dots of different shape and size (Fig -2a). The polar surface with markings showed dark spots with spackles, large and small dots are observed but the dots were very large dent like structures scattered on the shell surface (Fig-2b). More pores were also observed to the tip of egg. whereas the egg shell Giriraja fowl was thick compared to poultry chicken egg shell.
The quantitative analysis of protein by Lowry's method of four avian egg shell yielded reliable results with slight difference between them. The amount of protein present in 100mg of poultry chicken egg shell, native chicken egg shell, Giriraja egg shell and pigeon egg shell are 20µg/100mg, 150µg/100mg, 85µg/100mg and 250µg/100mg respectively as shown in (Table 1). The quantitative analysis by Lowry's method revealed the presence of more protein in pigeon egg shell and less protein in poultry egg shell, compared to Giriraja egg shell, the native egg shell contained more protein.
The Electrophoretic analysis of selected avian egg shell with membrane by SDS-PAGE method revealed the presence of protein as bands. The analysis revealed the presence of two kinds of protein bands in pigeon of 63.09K Da and 44.04K Da, poultry chicken egg shell too had two types of water soluble proteins of 21.98 KDa and 8.91 KDa one kind of protein band of 44.05KDa was recorded in native chicken and giriraja chicken egg shell too had produced single band for protein with molecular weight of 39.21Kda (Fig-3, Table2).

Table 1: Quantity of protein in different egg shell sample estimated by Lowry's method

Types of egg shell	Protein present in µg/ 100mg
Poultry chicken egg shell (*Gallus gallus domesticus*)	20
Native chicken egg shell (*Gallus gallus*)	150
Giriraja chicken egg shell	85
Pigeon egg shell (*Columbia livia*)	250

Table 2: Molecularweight of SDS protein electromorphs of different egg shell samples.

Samples	Migration distance	Rf value	Molecular weight in KDa
Poultry egg shell band1	4.1	0.585	21.98
Poultry egg shell band 2	6.3	0.9	8.19
Native egg shell	2.4	0.342	44.05
Giriraja egg shell	2.7	0.385	39.21
Pigeon egg shell band 1	1.4	0.2	63.09
Pigeon egg shell band 2	2.2	0.314	44.04

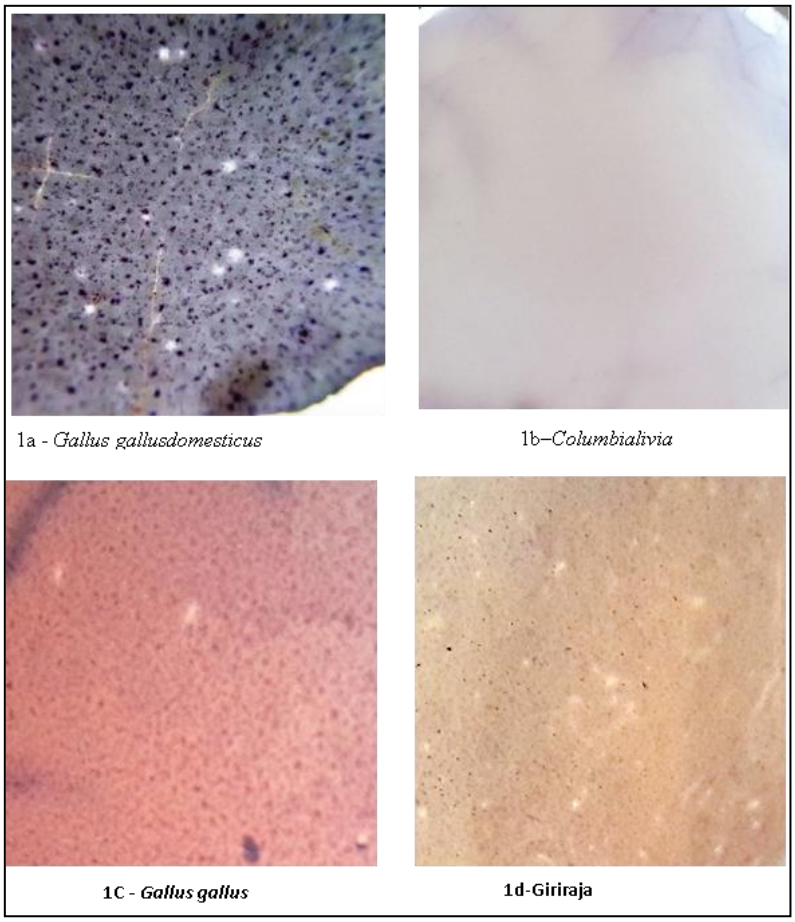


Fig 1: Ninhydrin stained eggshell photographs (1a-1d) shell pores visible as circular white spaces

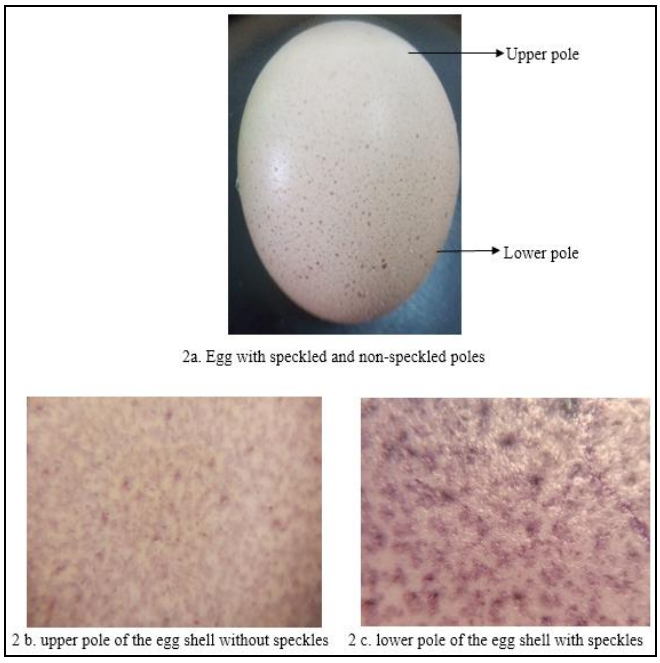


Fig 2: Giriraja egg showing different shaded regions - a.unstained whole egg, b and c Ninhydrin stained.

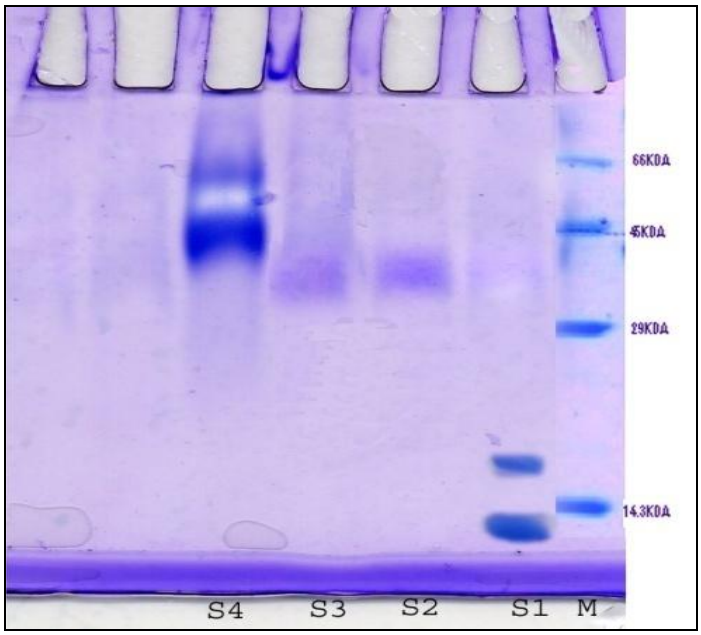
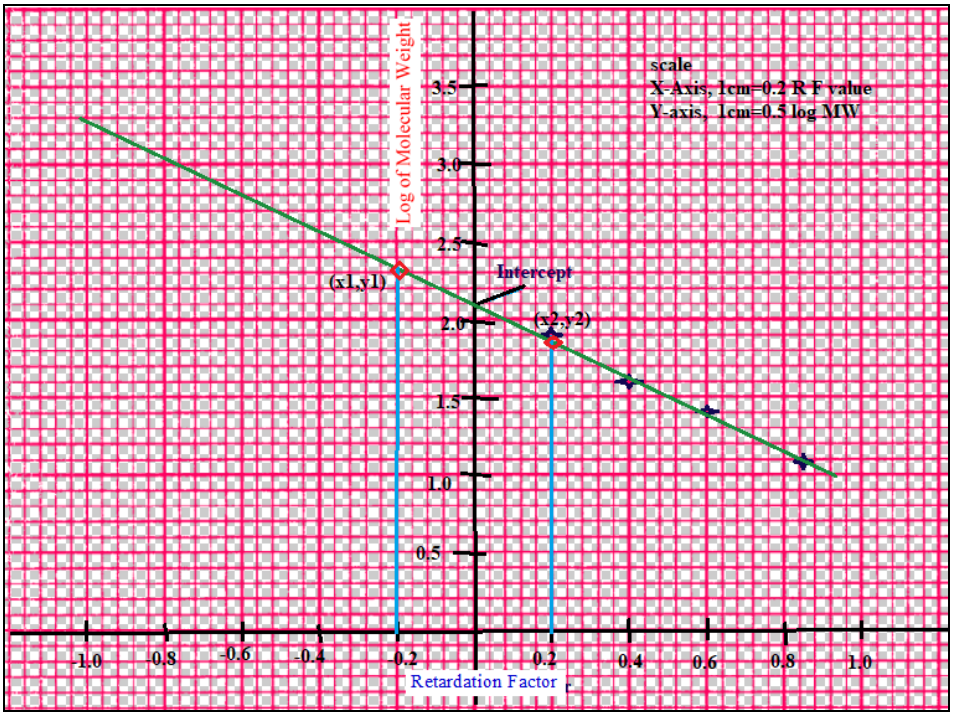


Fig 3: SDS PAGE Profile of egg shell proteins of different Egg species.M-Marker, S1 - *Gallusgallus domesticus*, S2 - *Gallus gallus*,S3-Giriraja and S4 –*Columbia livia*.



Graph 1: Standard curve to marker protein with known molecular weight

4. Discussion

Terrestrialization of vertebrates has led to many adaptive changes, one of those changes is oviparous reproduction in birds by producing cleiodioc eggs or hard shell covered eggs. The egg shell is necessary for propagation of all avian species by its perfect function in reproduction. Three basic function are performed by the egg shell - It acts barrier for physical environment and microbes, facilitates exchange of oxygen and water through its pores during the development of chick embryo [9, 10], it supplement calcium to the developing embryo [11].

Bird's egg shell display variety and pattern of color have been found to be results and two synthesized pigment biliviridin and protoporphyrin. The egg shell pigment are deposited at the end of the shell calcification process [12]. Egg shell coloration pure white was seen in the eggs of *G.gallus domesticus* and C.*livia* whereas the egg shell of the *G.gallus* and Giriraja had shown the golden brownish coloration. The color of brown and white egg shell are both results of the same pigment deposition at different rate with in the cuticle and outer calcified layer of the egg shell but the variation in shell colors are due to combination of light hues [13].

The presence of shell speckles or spots commonly found in brown eggs, from an evolutionary perspective this speckling is an adaptive trait most wild bird's species use it as camouflage to hide hatching eggs. In the evolution of modern chicken speckling had a selective advantage. Speckling has been successfully reduced with genetic selection and Shell color is an important trait to be studied due to different market preference [14] because choice of coloured and white egg varies among the consumers. The Griraja egg shell revealed the presence of two types in one type 3/4th of the shell had brownish speckles and 1/4th of the egg had plain non-speckle area. In the second type of egg, speckles were missing on the egg shell such an appearance of egg shell may be due to presence of two types of genic function or physiological function taking place during terminal phase of the egg shell mineralization in the oviduct because speckles and egg shell characters have been proved to be influenced by gene and are inherited [15]. This egg shell coloration and speckle formation have selective values. The Giriraja hen is a synthetic species produced in India by crossing exotic breed of chickens maintaining separate male and female lines through selection [16]. Therefore it is assumed that the brown color and speckles on shell are one of the linage characters expressed in this synthetic variety. This chicken majorly designed to produce more meat and large egg has successfully yielded both.

Gallus gallus domesticus and Giriraja egg shell contain more number of pores whereas in *Gallus gallus* less number of pores are seen for the given area on the egg shell but pigeon egg shell has minute pores which could not be recorded on photography due to least transparency of the shell membrane but appeared as dented structures under microscope. The shell pores gives porosity for ventilation of gas and water.The egg shell porosity is determined by three factors, number of pores, cross sectional area of pores and its length [17] The clusters of protein are more prominent in *Gallus gallus domesticus* but also present in both *Gallus gallus* and Giriraja with prominent small sized scattered proteins. But Giriraja has larger protein clusters in speckle region but medium sized in non-speckle area.

This results clearly implies that the egg shell of the three species of family galidae has more number of large size pores where as in *Columbia livia* proteins of Ninhydrin positive reaction was totally different indicating the absence of the type of clustered protein recorded in the other three birds. Thus Ninhydrin staining gives a clue for the existence of a type of protein on the egg shell or its absence. We strongly suggest this analysis could be used as an index test of shell morpology.

The protein being structural entities of evolution the nature and organization definitely will have an adaptive value for the egg shell [18]. The nonexistence of Ninhydrinpositive clustered protein on the surface of pigeon egg is curious enough for further analysis and determination of protein types in the shell.

The quantitative estimation of proteins of egg shell also revealed variation in protein quantity among the 4 species of eggs. Of this pigeon egg which had highest of 250µg proteins for every 100 mg shell while Giriraja had least quantity of 85µg of proteins/100mg of shell, poultry and native chick as

20µgand 150µg proteins for every 100 mg respectively. Thus the large clustered appearance of shell surface protein are not indicative of large quantity but only indicative of qualitative nature of protein existing on the shell surface. The amount of protein of egg shell revealed the industrial application of egg shell as a protein supplement is not ideal while its industrial application for calcium extraction is highly accepted [19].

Qualitative and quantitative proteomics studies have been used by many authors [20] to analyse different types of protein involved during mineralization of egg shell using oviducal fluid. About 216 types of shell matrix proteins were found be involved in four different phases of egg shell and its membrane formation. Mann [6] has worked on ovacleidin analysis of the egg shell. Gautron and Nys [3] have worked on the egg shell matrix protein that could have natural defense function of the egg these authors have recorded antibacterial protein lysozyme and ova transferring, ovacalyxin -36 and -25, during egg shell formation, therefore the shell and shell membrane protein have been considered to be one of the defensive mechanism of the egg shell. These workers have used only protein procured during shell formation whereas Miksik *et al*. [21] have analyzed other than water soluble components of protein in egg shell matrix. None of the authors have worked on the possible existence of the water soluble protein present in egg shell matrix. In nature interaction of egg shell with the moisture cannot be ruled out [22]. The same possible chance has influenced us in the analysis of water soluble protein present in the egg shell. Four different types of water soluble proteins have been recorded as electromorphs in the egg shell of galidae. Two protein types of 21.98 KDa and 8.91KDa were recorded in *Gallus gallus domesticus* that are different from the protein of 44.05KDa of *Gallus gallus* and 39.21 KDa of synthetic chicken Giriraja.Therefore molecular weight of 44.05KDa of *Gallus gallus* has to be considered primitive type followed by the Giriraja chicken that has retained only one type of shell matrix protein that has been selected during hybridization of more than two species of maternal and paternal lines,the selective advantage of this particular protein is of more interest that has to be confirmed by further analysis.

The species *Gallus gallus domesticus* compared to other type had protein of lesser molecular weight of 21.98KDa and the least of 8.91KDa therefore it has to be assumed among the three species the native chicken has more primitive type protein than the others. In total four genes are involved in the synthesis water soluble protein of in this three species of chicken -two genes in *Gallus gallus domesticus* -one gene in *Gallus gallus* and one gene in Giriraja. The profile of pigeon egg shell is entirely different it has two water soluble proteins represent as two electromorphs of 63.09 KDa and 44.04 KDa based on the molecular weight it could be assumed that pigeon egg shell has more primitive type than that of galidae. Further analysis of water soluble protein involving more number of egg species may yield good results to establish the phylogenetic relationship as well taxonomic categorization of the birds.

5. Conclusion

Avian egg shell is one of the many perfect evolutionary designs generated during terrestrialization of vertebrates. The shell provides protection to the growing embryo within. It also acts as ventilator and barrier between the inner and outer environment. The shell is not merely composed of calcium crystals but has integrated many proteins in it. The quality and quantity of the integrated proteins have been analyzed in this study, revealed variations between each of the shell type. This difference in water soluble proteins of eggshell and ninhydrin positive proteins seems to be of taxonomic and evolutionary significance that has to be examined.

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