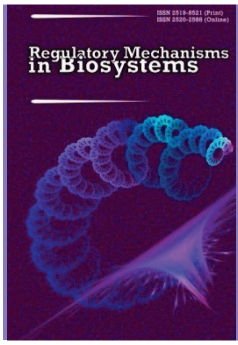

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Morphological features of the cuticle of hatching eggs of chickens and turkeys subjected to pre-incubation treatment

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Improving the productivity of poultry, especially egg crosses, depends on the quality of the bioceramic calcite ($CaCO_3$) layers and surface glycoprotein film of the egg (cuticle). These are the barriers that control the flow of gases and water vapor. Any disruptions in the transport process can lead to a significant reduction in egg hatchability. It has been proven that treatment of chicken and turkey eggs with acid solutions (acetic acid or hydrochloric acid) and sodium hypochlorite prior to hatching has increased egg hatchability compared to the control. In view of this, the aim of this study was to investigate the protective structures of incubation eggs of chickens and turkeys using non-destructive electron microscopic methods and computer processing of digital images of the cuticle coatings of these eggs for reliable prediction of the degree of increase in gas permeability of the cuticle-shell system due to the destructive effect of various chemicals, including disinfectants, for pre-hatching egg treatment. In the experiments, hatching eggs of Leghorn White hens (n = 90) 15–20 weeks of laying and Broad Breasted White turkeys (n = 80) were used. The antimicrobial agents used in the experiments were a 0.6% solution (0.08 mol/L) of sodium hypochlorite, a 2.5% solution (0.4 mol/L) of acetic acid, and a 5.0% solution (1.40 mol/L) of hydrochloric acid. Using the computer analysis of digital electron microscopic images of the egg surface with the software packages Visilog and FemtoScan Online, it was experimentally proved that the destructive effect of these substances on the cuticle-shell system increases in the range of sodium hypochlorite < hydrochloric acid < acetic acid, which positively correlates with the gas permeability of hatching eggs of chickens and turkeys and the egg hatchability index. The presence of fundamentally different morphological features and correlations of the cuticle of chicken and turkey eggs in response to the action of acids and oxidants used for pre-hatching treatment was shown. The digital markers of the cuticle-shell system state, obtained from analytical programs of digital images, have been established, which makes it possible to reliably predict the indicators of increasing the hatchability of chicken and turkey eggs under the conditions of using certain chemicals. The prospect of further research is to study the effect of modern complex antimicrobial agents on the hatchability of poultry eggs of different species.

Keywords: technology; air temperature; live weight; average daily gain; clinical parameters.

Introduction

The artificial breeding of young poultry through incubation is one of the main links in modern poultry production technology (Zhong et al., 2018; Hedlund & Jensen, 2021). The quality of eggs used for incubation is the most crucial and essential factor in accurately forecasting the hatchability and viability of offspring and acquiring safe and excellent poultry products (Goliomytis et al., 2015; Melo et al., 2021). It should be noted that the quality of hatching eggs is influenced by an array of biotic and abiotic factors (Nasri et al., 2020; Orobchenko et al., 2022).

Before incubation, eggs are sorted, their weight, shape, condition, and quality of the shell are determined, and attention is paid to the size and position of the air chamber, the position and mobility of the yolk, and the presence of inclusions (Adegbenjo et al., 2020; Mesquita et al., 2021).

An important and integral stage in the overall complex of veterinary and sanitary measures aimed at preventing the occurrence of infectious diseases and reducing bacterial contamination of poultry facilities is the pre-hatching treatment of poultry eggs of various species (Olsen et al., 2017; Rehkopf et al., 2017). For the treatment of hatching eggs, substances with biocidal activity are used (Chung et al., 2018). For this purpose, alkalis, acids, oxidizers, chlorine and aldehyde preparations, and antibiotics are used (Sylte et al., 2017; Paliy, 2018; Tebrün et al., 2020). It should be noted that the treatment of hatching eggs with acids and oxidants has the purpose, in addition to exerting biocidal effects on harmful pathogens, of stimulating the metabolic rate of the developing embryo by improving transshell active and passive (diffusion) gas transport, first of all, oxygen, water vapor and carbon dioxide, which leads to significant increase in such an important indicator of incubation success as egg hatchability, which in turn increases the yield of healthy young birds (Breslavets et al., 2006). Both chemicals and physical factors have been successfully used for such stimulation (Wlazlo et al., 2020; Abuoghaba et al., 2021). Such modifications of commonly used poultry egg incubation technologies are used primarily for incubating eggs of bird breeds and crosses that have a naturally low hatchability rate, such as meat chicken breeds, or for farms that, due to force majeure, cannot ensure that poultry are kept in conditions for preserving hatching eggs which are in accordance with established standards. A clear example of the latter is the conditions of poultry farming in farms located in areas near the hostilities in eastern Ukraine. Taking into account all of the above, as well as the latest data on the much greater contribution of the cuticle cover in the cuticle-shell defense system to the protection of eggs from contamination by harmful pathogens and to modulation of transshell transfer of gases and water vapor during incubation, the aim of this study was to investigate the protective structures of incubating eggs of chickens and turkeys using non-destructive electron microscopic methods and computer processing of digital images of cuticle coatings of these eggs for reliable prediction of the degree of increase in gas permeability of the cuticle-shell system due to the destructive effect of various chemicals for pre-hatching treatment of eggs on the components of the cuticle, on the example of acetic and hydrochloric acids, as well as sodium hypochlorite as a typical powerful oxidizing agent.

Materials and methods

The research was conducted in the period from 2015 to 2022 at the Clinical-Diagnostic Centre of the Faculty of Veterinary Medicine and the Department of Biochemistry and Biotechnology, Sumy NAU, and Department No. 20 of the Institute of Applied Physics of NAS of Ukraine, Sumy, Ukraine. Treatment of hatching eggs with chemicals and preparation of samples for research was carried out in the Laboratory of Veterinary Sanitation and Parasitology of the National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine", Kharkiv, Ukraine.

The study employed hatching eggs from 90 White Leghorn hens and 80 Broad Breasted White turkeys aged between 15–20 weeks of egg-laying. The eggs were obtained from poultry kept in accordance with generally accepted standards of housing and feeding.

The study of the shells of hatching eggs of chickens and turkeys (morphological and functional parameters) was carried out both in the norm (control) and after their treatment with aqueous solutions of the following substances:

– 0.6% solution (0.08 mol/L) of sodium hypochlorite;
– 2.5% solution (0.4 mol/L) of acetic acid;
– 5.0% solution (1.40 mol/L) of hydrochloric acid.

Egg hatchability and gas permeability of the cuticle-shell system were determined in accordance with existing recommendations (Breslavets et al., 2006).

The structural characteristics of the shells were studied using a scanning electron microscope (scanning electron microscopes with X-ray microanalyser "Remma-102" and "Remma-106i" (Selmi, Sumy, Ukraine). Digital images of biocrystalline layers were processed using the Visilog 6.11 software package (Noesis, 2000, trial version). The electron microscopic images were digitized onto a 320 × 320 pixels matrix (magnification × 250) with a grey level of 0–255. The digital images of the egg surface were analyzed using the "line" and "profile" functions in the digital image analysis package Visilog 6.11. In order to quantify the microdefects of the shell surface layer in normal conditions and under chemical treatment, these digital images were subjected to image processing and analysis using the FemtoScan Online Program software package (Advanced Technologies Centre). The Visilog 6.11 software package enables one to obtain a surface profile of a digital image obtained by a scanning microscope. The FemtoScan Online Program package also makes available a number of parameters characterising the profile roughness, namely: R_a – average roughness (A), R_{max} – maximum profile height (B), R_z – height of profile irregularities by 10 points (C), R_q – root mean square roughness (D), R_{sk} – asymmetry parameter (E), R_{ku} – measure of excess (F) (Fig. 2). Working distance (WD) – defined as the distance between the focused surface of the sample and the edge of the objective lens (electron output). It is decisive for the obtained resolution, signal-to-noise ratio, depth of field, and the obtained magnification (viewing magnification). The voltage of the device is 20.00 kV. The prototypes were studied at magnifications of ×100, ×200, ×300, ×500. The bars of the presented objects are 100, 200, 500 μm. In the graphs (digital profiles), pixels (px) are represented on the X-axis, and the Y-axis shows the increase in the brightness of pixels belonging to the area under study.The results were statistically processed using the OriginPro 2021 package.

Results

It has been proven that spraying eggs with aqueous solutions of acetic and hypochlorous acids and sodium hypochlorite solution has a significant stimulating effect on both the level of gas permeability and the egg hatchability index derived from this parameter. At the same time, the greatest positive effect on eggs of both chickens and turkeys belongs to acetic acid – 94.3% (chickens) and 85.9% (turkeys) of the controls in terms of hatchability (89.8% and 78.6%, respectively). The treatment of eggs with an aqueous solution of hypochlorous acid has a similar, but less pronounced effect on the hatchability, 93.6% (chickens) and 83.1% (turkeys). As for the effect of sodium hypochlorite, this substance stimulates the hatchability of eggs in chickens and turkeys in a similar way: 91.1% (chickens) and 80.0% (turkeys) with the corresponding gas permeability indicators of 110% (chickens) and 117% (turkeys).

Generally, the order of acids and sodium hypochlorite with regard to egg production stimulation in chickens and turkeys can be arranged as follows: acetic acid > hydrochloric acid > sodium hypochlorite.

The digital electron microscopic images of the control chicken and turkey shell samples (Fig. 1 and 2) show a natural, well-defined cuticle, which is a dried protein film on the surface of the solid phase (in this case, the surface crystalline layer of the shell). The white areas indicate the accumulation of charges near the crack, an artifact that is an inherent characteristic of this physical method of digital imaging.

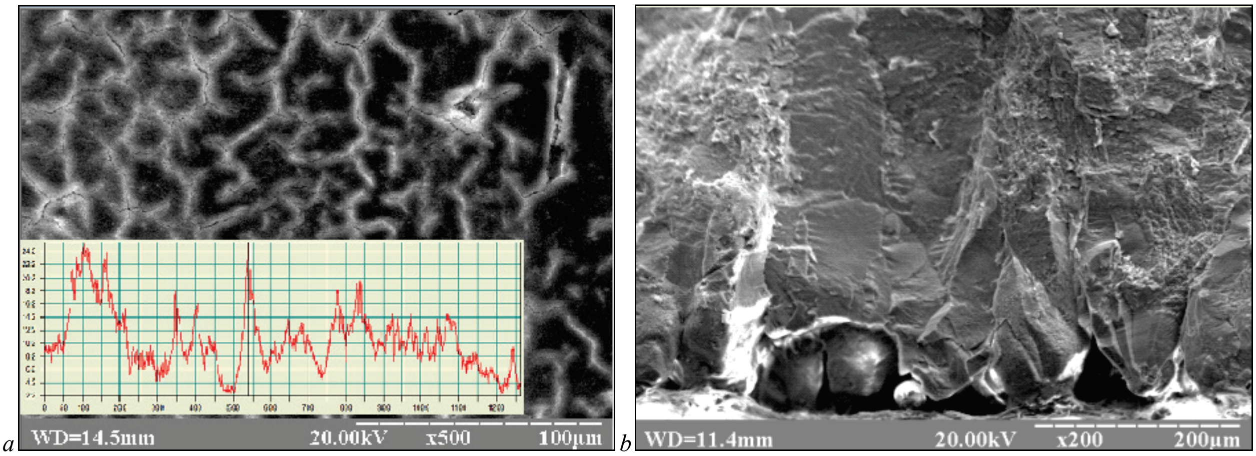

Fig. 1. Electron microscopy of a White Leghorn chicken egg (control): *a* – cuticle surface (*a* – general view of the surface; here and in the following images, the bottom left shows the surface profile of the cuticle layer of the shell obtained using the Visilog 6.1 software package); *b* – shell section; n = 15

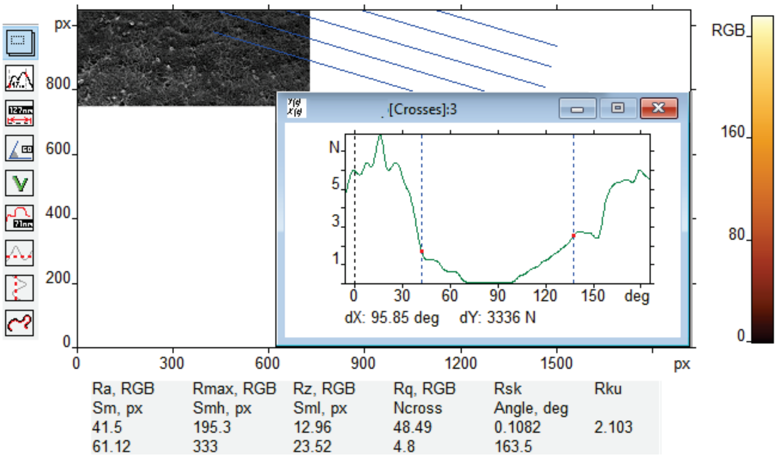

Fig. 2. Electron microscopy of a White Broad Breasted turkey egg (control): the upper corner shows an image of the cuticle surface; the right corner shows an image of the cuticle surface profile of the shell layer and the table below shows the values of quantitative indicators characterising the morphological features of the cuticle surface obtained using the FemtoScan Online software package

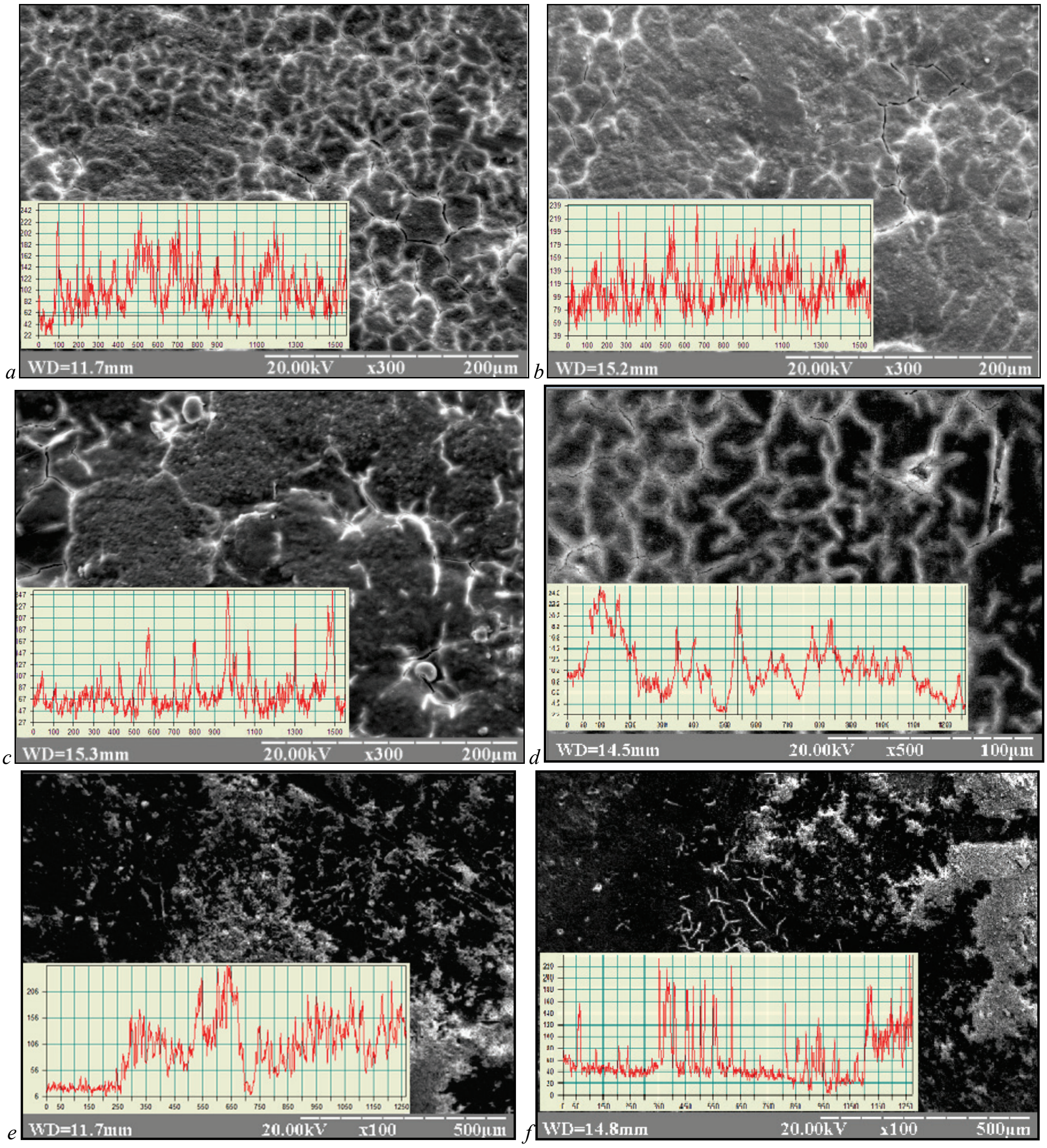

Fig. 3. Electron microscopy of the surface of the cuticle layer of the shell of a White Leghorn chicken egg (*a* – treatment with 0.6% sodium hypochlorite solution, n = 25; *b* – 5.0% hydrochloric acid solution, n = 25; *c* – 2.5% acetic acid solution, n = 25) and White Broad Breasted turkey eggs (*d* – treatment with 0.6% sodium hypochlorite solution, n = 20; *e* – 5.0% hydrochloric acid solution, n = 20; *f* – 2.5% acetic acid solution, n = 20)

Table 1
Correlations between the degrees of gas permeability and hatchability of hatching eggs of chickens and turkeys and parameters of surface roughness of the cuticle layer of control and treated eggs (n = 170)

Eggs	Drug	Egg hatchability, %	Gas permeability, % of control	A	B	C	D	E	F
Chicken	Control	89.8 ± 0.04	100	42.6	355.1	88.1	55.1	0.0377	4.058
	Sodium hypochlorite	91.1 ± 0.02	110	50.3	320.1	92.8	66.1	-0.2298	3.045
	Hydrochlo-ric acid	93.6 ± 0.02	159	53.9	330.6	109.6	65.7	-0.0211	2.862
	Acetic acid	94.3 ± 0.06	180	60.1	375.3	113.8	72.4	0.1570	2.671
	r- Pearson			0.93075	0.44897	0.99513	0.81064	0.59562	-0.80923
	Correlation *p*-value			0.069	0.551	0.004	0.189	0.404	0.190
Turkey	Control	78.6 ± 0.02	100	59.3	291.9	69.11	70.6	-0.2757	2.153
	Sodium hypochlorite	80.0 ± 0.04	117	62.8	317.6	64.2	76.0	-0.4194	2.412
	Hydrochlo-ric acid	83.1 ± 0.05	137	54.1	287.8	93.3	68.2	-0.5301	3.073
	Acetic acid	85.9 ± 0.02	163	47.7	269.0	90.8	58.0	-0.1641	2.327
	r- Pearson			-0.88791	-0.68788	-0.81207	-0.83148	0.27233	0.30026
	Correlation *p*-value			0.112	0.312	0.187	0.168	0.702	0.690

Note: the values of Pearson correlations presented in the table, characterise the correlations between the gas permeability of the shell of eggs of chickens and turkeys and the parameters of the surface roughness of the cuticle layer.

We examined the cuticle-shell system of chicken and turkey eggs post-treatment with solutions containing substances that promote the transport of gases and water vapor through the egg shell and that have some antimicrobial properties: sodium hypochlorite, acetic acid and hydrochloric acid (Fig. 3). It has been demonstrated that the profiles of the cuticle surface in digital images (Visilog 6.11 software package) exhibit some differences with the control, which is most relevant for eggs treated with acetic acid, both for chickens and turkeys, but these differences are purely empirical and in this case cannot be characterised by quantitative parameters (Fig. 3c, 3f). The cuticle cover profile of chicken and turkey eggshells treated with sodium hypochlorite solution showed no significant differences from the control sample. This indicates that hypochlorite does not have a substantial destructive effect on the morphological structure of the cuticle surface (Fig. 3a, 3d). The same result was observed for eggs treated with hydrochloric acid solution (Fig. 3b, 3e).

Thus, using only computer profile construction technology on digital images of the cuticle, and even more so, the crystalline calcite layer of the shell, does not offer the possibility of obtaining quantitative data that accurately corresponds with the indications of gas permeability and egg hatchability (Fig. 1–3). The FemtoScan Online Program, a software package for precision surface sensing of solid and semi-solid samples, enables the acquisition of various parameters that describe profile roughness.

The analysis of the surface profiles of control and treated chicken and turkey eggs shown in Figures 1–3, made it possible to establish correlations of different power and direction between the parameters of roughness and the degrees of gas permeability of the cuticle-shell structures (Table 1). It was found that the parameters characterizing the roughness of the cuticle surface in the cuticle-shell system of chicken and turkey eggs are fundamentally different: parameters A, B, C, and D are positively correlated with the gas permeability and hatchability of eggs. The closest direct correlation is inherent in indicator C (height of profile irregularities by 10 points): r –0.99513, p-value 0.004 and A (average roughness): r –0.93075, p-value 0.069. For indicator D (RMS roughness), a less close positive correlation was observed: r –0.81064, p-value 0.189. As for turkey eggs, they are characterized by a fundamentally different type of correlation between the values of gas permeability and egg hatchability and the morphological parameters of the cuticle-shell structures. Thus, the values of indicators A, B, C and D of the cuticular coatings of turkey eggs, in contrast to the same indicators of chicken eggs, negatively correlate with the indicators of gas permeability and egg hatchability, and the degree of correlation is low (Table 1).

Discussion

Today, the poultry industry plays a key role in providing the population with food of animal origin. The success of sustainable development of this industry depends on a number of factors, both national and technological (Ben Sassi et al., 2016). The leading importance is given to ensuring the epizootic welfare of the livestock (Kennedy et al., 2017; Bogach et al., 2021). The main technological stage in the development of the poultry industry has been and remains incubation, which involves a number of labor-intensive and routine technical and technological operations (Albokhadaim et al., 2010; Clark et al., 2017; Riaz et al., 2021). Along with the correct selection of eggs, the correct and scientifically based use of egg disinfectants before incubation is of paramount importance (Oliveira et al., 2020). For this purpose, a number of chemicals are used that have a wide range of biocidal effects and are widely used in practice (Niu et al., 2017). In addition, such chemicals indirectly increase egg hatchability by increasing the gas permeability of the egg's cuticle-shell defense system. The quality of eggs intended for incubation is the most important indicator for reliably predicting hatchability and viability of offspring. The purity and integrity of the shell is of great importance (Sozcu & Ipek, 2015; Moriyama et al., 2021).

The eggshell is a rather complex multicomponent bioceramic shell that directly borders the external environment and performs several functions: protective and gas exchange. Due to mineralization, the shell is characterized by high strength with a small thickness (up to 300 µm) (Solomon, 2010). Biomineralization is a unique process of forming bioceramic structures consisting of layers of biomacromolecules, in particular peptides and proteins, in a layer of inorganic substances, mainly calcite. In most cases, bioceramic materials are formed on a kind of bimolecular scaffold of molding peptides and small proteins (DeKetelaere et al., 2002; Ha et al., 2007). It has been proven that, if the shell is not damaged, the movement of bacteria and viruses inside hatching eggs is limited by two most important factors: a thick, well-structured cuticle layer and the absence of water in the external environment (Jones & Musgrove, 2005; Ramzan et al., 2020). It has been proven that if hatching eggs are heavily contaminated with organic matter, in particular feces, the use of disinfectants is not effective, as feces adsorb and neutralize the components of the drugs (Melo et al., 2019). For example, Rehkopf et al. (2017) observed a significant increase in gas permeability of chicken eggshells when eggs were treated with a 2.5% acetic acid solution after one minute. In 5 minutes after treatment, the vapor permeability of the shell increases almost twice, which at the end of the incubation process leads to an increase in egg hatchability by 10–15%.

Based on the research, it can be concluded that chemicals from a number of acids and oxidizing agents (after drying on the treated egg surface) to some extent affect the morphology of the natural cuticle, but do not completely destroy it. For example, sodium hypochlorite has a harmless effect on the cuticle surface, but contributes to an increase in gas and vapor permeability of the entire surface of the eggshell of chickens and turkeys.

However, the latest data (Attard et al., 2023) on the cuticle of poultry eggs as a complex heterogeneous biocomposite formation of organic and inorganic composition prompted us to conduct an in-depth analysis of the structural and functional relationships of the cuticle-shell structure in order to improve the technologies of pre-hatching treatment of poultry eggs used in industrial poultry farming in Ukraine, in particular chickens and turkeys. The scientific basis for such studies was shown (D'Alba et al., 2016; Kulshreshtha et al., 2022; Lee et al., 2023) to be a significant structural and functional difference between the cuticular coatings of eggs of different poultry species.

An informative and accurate research method in veterinary medicine is the use of a scanning electron microscope (Madkour & Abdelsabour-Khalaf, 2022). Thus, it should be noted that the tested acids and oxidants, according to electron microscopy, do not have a pronounced destructive effect on the calcite layers of the shell, except for the upper so-called crystalline layer, but increase the number of breaks on the cuticle surface and increase its density between breaks. In this case, the increase in air and vapor permeability of the shell surface is observed in places of local cuticle damage. Our results substantiate the data obtained by Breslavets et al. (2006) that acetic and hydrochloric acid solutions increase the air permeability of the shell by 1.7 and 1.9 times, respectively, and its vapor permeability by 1.5 and 2.0 times, but the detailed molecular mechanism of increasing gas and vapor permeability has not been determined. On the basis of computer analysis of digital electron microscopic images by various software packages, in particular Visilog 6.11 (Noesis) and FemtoScan Online Program (Advanced Technologies Center), it was proved that the treatment of the surface of chicken and turkey eggs with solutions of sodium hypochlorite and acids (acetic or hydrochloric) differs in the mechanism of action and the severity of the destructive effect mainly on the organic layers of the cuticle in the cuticle-shell protection system and on nanoscale globular formations of hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ (D'Alba et al., 2016), which are part of the organic, predominantly glycoprotein cuticle matrix. Although, in general, the destructive effect decreases in the order acetic acid > hydrochloric acid > sodium hypochlorite and correlates with the values of gas and vapor permeability of the cuticle-shell structure of incubated chicken and turkey eggs, it should be taken into account that, as shown by (Chen et al., 2019), it is the cuticle coatings of chicken and turkey eggs that are fundamentally different in size and spatial arrangement of nanoscale globular formations of hydroxyapatite in the cuticle matrix (D'Alba et al., 2016). The difference consists, in particular, in the fact that the average diameter of these formations is significantly higher than the corresponding parameters of such formations in the cuticle of chickens (Joonbum et al., 2023), and this difference may cause a significant difference between the ability of pathogenic bacteria to penetrate the cuticle-shell barrier of a bird egg. For example, Salmonella is found in turkey eggs in much smaller quantities compared to chicken eggs: 3.5% vs. 15.6% (Chen et al., 2019). Since the above is the basis for reasonable conclusions about the importance of structural and functional parameters of the cuticle in controlling the kinetic parameters of gas and vapor permeability of the shell and indirectly the state of protection of the egg against secondary contamination with pathogenic microflora, we recommend conducting preliminary screening of chemicals for preparations for pre-hatching treatment of poultry eggs of different species, breeds and even crosses by examining digital images of cuticular coatings of hatching eggs using the software packages that include functions for calculating surface roughness parameters (Attard et al., 2023). The roughness of a surface refers to its waviness (height) and unevenness and affects the functionality of the surface – not only surface properties such as hydrophobicity, adhesion, and friction, but also bulk properties such as fracture toughness and mechanical stability. In nature, there are numerous examples of smooth and rough surfaces, each of which performs a specific function. Surface roughness also affects the ability of water droplets to attach to the surface, and thus affects the adhesion and removal of bacteria (Attard et al., 2023). The authors state that the surface roughness of the cuticle of chickens differs significantly from that of turkeys due to the fact that they contain nanoscale spherical formations of hydroxyapatite of different shapes and spatial arrangements, which react with different intensities to acids and oxidizing agents.

Thus, the equilibrium constant K_c for the reaction of hydroxyapatite with hydrochloric acid is 2.9×10^{16}, greater than one, which indicates a high degree of process completion, and the lowest for the reaction of hydroxyapatite with acetic acid is 1.4×10^{-17}, less than one, which indicates the impossibility of its completion under standard conditions. Thus, acetic and hydrochloric acids affect the main inorganic component of the cuticle to varying degrees, and hydrochloric acid has the most damaging effect. Sodium hypochlorite, as a typical peroxide, has almost no effect on the mineral part of the cuticle, but destroys mainly its protein components.

Conclusion

Thus, peroxide compounds and solutions containing organic acids with low concentrations (mainly acetic acid), which have the minimum detrimental impact on the inorganic and organic constituents of the cuticle of poultry eggs as a complex biocomposite, could be considered as certain prospects in the development of new drugs for the pre-incubation treatment of chicken and, in particular, turkey eggs. As for hydrochloric acid, it, like other powerful inorganic acids, can be used only in some cases in the technology of incubating waterfowl eggs (geese, ducks, etc.), whose egg cuticles are characterized by increased mechanical resistance and are much thicker than the corresponding formation of birds living on land.

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