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Effects of *Histomonas*, *Trichomonas*, and *Eimeria* Co-infection on Productivity and Macro-morphological Indicators of Eggs in Laying Hens

Petro Liulin[1*], Mykola Bogach[2], Liubov Lyakhovich[1], Alla Petrenko[1], and Inna Kostyuk [1]

[1]*State University of Biotechnology, 44, Alchevskikh Str., Kharkiv, 61002, Ukraine*
[2]*Odessa Research Center, National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine" Nacional Academy of Agrarian Sciences of Ukraine, Odessa, Ukraine*

*Corresponding author's Email: liulinpetr@gmail.com

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ABSTRACT

The study of macro-morphological changes is important for recognizing disturbances in egg formation that cause pathologies, especially co-infection. The current study aimed to evaluate the level of egg productivity and macro-morphological parameters of eggs in domestic chickens of the Rhode Island breed with co-infection of *Histomonas*, *Trichomonas*, and *Eimeria*. Clinical and parasitological, coproscopic, morphometric research, and statistical analysis methods were used for this research. Pathogens of *Histomonas* and *Trichomonas* were detected by microscopy of smears of fresh feces, and *Eimeria* oocysts were identified by flotation according to the Fullenborn method. During 30 days of research, there was a significant decrease in egg production (52%), a decrease in egg weight by 16.8%, and a decrease in the shell thickness by 30.43% during spontaneous *Eimeria-Histomonosis-Trichomonosis* co-infection in laying hens. The eggshell indicated noticeable macro-morphological changes, including deformations and defects resulting from insufficient calcification. These changes manifest as combined damage to the shell, characterized by small cracks, roughness, bumpy or spilled thickenings, and complete or partial depigmentation. When evaluating the internal content of eggs in 12% of their samples, there were bloody spots, relatively smaller and lighter yolks, thinning of the protein part. Thus, the specified macro-morphological changes and egg defects were the result of the negative impact of co-infection on the processes of egg formation, which indicates the systemic nature of the lesion and the morphofunctional insufficiency of the egg-forming organs.

Keywords: Comorbidity, Egg defect, Egg production, Eimeriosis, Histomonosis, Laying hen, Trichomoniasis

INTRODUCTION

Recently, protozoan diseases, particularly avian *Eimeria*, have emerged as a significant obstacle to increasing demand for chicken meat and egg production, as recognized by the United States Department of Agriculture (Godfray et al., 2010; USDA, 2023). In terms of importance, this disease is one of the top three (Dalloul and Lillehoj, 2006). The global poultry industry economy loses more than 14.5$ US billion annually (Blake et al., 2020). In adult chickens, cases of protozoan infestations (eimeriosis, histomoniasis, trichomoniasis) became more frequent (Dolka et al. 2015). They have become widespread in most countries of the world, particularly following the prohibition of protistocidal drugs (CEC, 2002), such as nitroimidazoles, nitrofurans, and arsenic drugs (Hess et al., 2015). Enterohepatitis of *Histomonas* and *Trichomonas* etiology (Dolka et al., 2015) and granulomatous liver lesions (Araújo et al., 2015; Lopes et al., 2022) are often detected in adult chickens. Despite being asymptomatic, these conditions are characterized by signs of a decrease in the reproductive capacity and periodic manifestations of diarrhea symptoms (Mehlhorn, 2016). Protozoan diseases significantly threaten the health of chickens (Chen et al., 2022; Tuska-Szalay et al., 2022; Saikia et al., 2023). They are widely distributed worldwide among different species of agricultural and wild bird species (Badparva et al., 2020). The prevalence of trichomoniasis epizootics among wild birds of ecoparks (Fadhil et al., 2020) often leads to a significant decrease in their populations and even threatens the disappearance of certain bird species in natural ecosystems (Forzán et al., 2010; Feng et al., 2021). Possible variants of cross-infection of birds with causative agents of trichomoniasis and histomoniasis from wild birds and vice versa have been proven (Tuska-Szalay et al., 2022). The development of *Eimeria*, *Histomonas*, and *Trichomonas* co-infection in adult chickens leads to chronic inflammation of the intestine, resulting in damage to the liver (necrotic hepatitis) and cecum (diphtheria typhitis) as noted by Shchebentovska and Holubtsova (2020). Spontaneous trichomoniasis in birds involves damage to the oral cavity, pharynx, small and large parts of the intestines, and the formation of granulomas with localization in the liver and cecum (Landman et al., 2019). According to

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Liulin et al. (2023), during co-infection eimeriosis, histomonosis, and trichomonosis in domestic chickens, lesions extended to other organs and tissues, including spleen, bursa of Fabricius, peritoneum, and even skin. The causative agents of avian trichomoniasis can cause damage to the organs of the reproductive system of birds, resulting in reduced or halted egg production (Falkowski et al., 2020). The productivity of chickens and a decrease in egg production and egg mass are also significantly affected by invasions of *Eimeria* spp. and *Histomonas meleagridis* (Dolka et al., 2015; Vakili et al., 2021), which needs further study. With this in mind, this study aimed to determine the level of egg productivity and classify macromorphological changes in the eggs of domestic laying hens of the Rhode Island breed with a spontaneous combination of eimeriosis, histomoniasis, and trichomoniasis infection.

MATERIALS AND METHODS

Ethical approval

The present study was conducted in compliance with the ethical norms and principles of scientific research specified by the European Convention on the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes. The approval of the Bioethics Commission of the State Biotechnology University (SBTU), Ukraine, was not required since the object and material of the study were the population of adult laying hens and their freshly laid eggs.

Stages of research

At the first stage of the research, a comprehensive examination was conducted, including general clinical, parasitological, and special coproscopic assessments of laying hens. A population of laying hens (n = 254) of the 1.5-year-old Rhode Island breed of a private farm in the Kharkiv region with a free-range organic housing system was investigated. Based on the results of the examinations, an experimental group (n = 35; average chicken weight of 2.20 ± 0.062 kg) with spontaneous *Eimeria-Histomonosis-Trichomonosis* co-infection and a control (n = 35; average chicken weight 2.64 ± 0.056 kg) group of non-infested laying hens were selected. The chickens were placed in separate sections of the room with a maximum density of 6 hens per 1 m^2. They were provided free range with a pasture area of 10 m^2 per head. The lighting schedule involved a 13-hour day, with an illumination level of 20 lux at the feeders. The air temperature in the poultry house was +18°C, and the relative humidity was 70%.

Coproscopic studies

Smears were prepared from fresh feces (immediately after defecation) to identify the causative agents of Histomonas and Trichomonas. These smears were then fixed with methyl alcohol for 3-5 minutes and stained according to the Romanovsky-Hiems method. To identify *Trichomonas*, the smears were air-dried and stained with methylene blue. Pathogens were identified by morphological features (Menezes et al., 2016) using light microscopy on an Axioscop-40 microscope (Zeiss Germany), magnification ×400.

Flotation method

Eimeria oocysts were detected following the Fulleborn flotation method (Halat et al.,2004) . Individual fecal samples were collected, primarily during defecation. Feces (3 g) were placed in a 100 ml glass, and a saturated solution of NaCl in a ratio of 1:20 was added while stirring with a glass rod. The resulting suspension was filtered through a metal filter (hole size 0.8-1.0 mm) into similar cups and left for 30 minutes. After settling, 3 drops of the surface film were collected using a metal loop (0.8 cm in diameter). These drops were then transferred to a glass slide and subjected to microscopy for the presence of *Eimeria* oocysts. Invasion intensity (the number of oocysts in 1 g of feces) was determined according to the McMaster method (Vadlejch et al., 2011). The species of *Eimeria* oocysts was determined according to Pellerdy (1974).

Accounting of egg productivity and macromorphological indicators of eggs

The productivity of experimental and control groups of laying hens was recorded daily for 30 days. The gross collection, the average laying capacity, and the number of rejected (%) eggs were recorded. Evaluation of macromorphological changes in eggs, and their analysis was carried out visually. Egg mass was determined by weighing on an Adventurer electronic laboratory scale. The egg shell thickness was examined using an ultrasonic thickness gauge (from 0 to 300 mm) Co. Ltd., Echometer 1061 Co. Ltd, Karl Deutsch, Wuppertal, Germany (Tsaruk and Dikhtiaruk, 2014).

Statistical analysis

Statistical processing of the obtained results was carried out using the descriptive statistics tool. For comparing the data, the Data Analysis package in MS Excel 2019 was utilized, specifically utilizing the two-sample t-test with different variances and correlation analysis. The average values of the main feature, including egg mass, were determined. Mean

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error and absolute error at a given confidence level p > 95%, which corresponds to the level of statistical significance p < 0.05 according to the Student's test (Lebed'ko et al., 2022).

RESULTS

The results of intravital clinical-parasitological and special coproscopic studies of laying hens of the Rhode Island breed (n = 254) indicated the spontaneous *Eimeria-Histomonosis-Trichomonosis* co-infection (EI-23.22%). Co-infection was caused by pathogens *Eimeria acervulina* (19.4%), *Eimeria brunetti (*7.9%), *Eimeria maxima (*16.5%), *Eimeria mitis* (5.1%), *Eimeria necatrix* (12.5%), *Eimeria praecox* (3.2%) and *Eimeria tenella* (35.4%), at the intensity of 49.6 ± 4.8-106.4 ± 5.7 oocysts in a gram of feces and *Histomonas meleagridis* and *Trichomonas gallinae* at intensity 1-3 of the pathogen in the field of view of the microscope (p < 0.05). Table 1 shows the results of the effect of *Eimeria-Histomonosis-Trichomonosis* co-infection on the level of egg productivity and egg culling of the experimental and control groups for 30 days.

Compared to the control group, egg production decreased by 52% in the chickens of the experimental group with spontaneous *Eimeria-Histomonosis-Trichomonosis* co-infection during the observation period (30 days). In addition, due to structural defects of the shell, 58.57% of eggs obtained from hens of the experimental group were rejected. Morphometric indicators of eggs obtained from experimental and control groups of chickens and the results of statistical processing are presented in Table 2.

At the same time, it was found that all morphometric egg parameters of the control and experimental groups differed significantly. Tables 3 and 4 tabulate the correlation coefficients of the morphometric indicators of eggs in the experimental and control groups.

Table 1. Indicators of productivity and hatching of Rhode Island chickens' eggs for 30 days

Indexes	Research group (n=35)	Control group (n=35)
Productivity for 1 laying hen in 30 days (eggs)	12	25
The average egg mass (g)	51.70	62.14
Gross collection of eggs for 30 days (pieces)	420	875
Eggs culled (%)	58.57	2.23

Table 2. Results of primary processing of morphometric parameters of Rhode Island chicken eggs

Statistical indicators	Average egg mass (g)	Egg shell thickness in the middle part (mm)	Egg shell thickness at the sharp end (mm)	Egg shell thickness at the blunt end (mm)
Control group (n = 35 chickens)				
M	62.14	0.322	0.337	0.322
M	0.24	0.001	0.002	0.001
ΔM	0.49	0.002	0.004	0.002
s^2	2.01	$5.176 \cdot 10^{-5}$	0.000129	$5.176 \cdot 10^{-5}$
Experimental group (n = 35 chickens)				
M	51.70	0.224	0.237	0.227
m	0.49	0.005	0.005	0.005
ΔM	1.00	0.010	0.011	0.009
s^2	8.41	0.001	0.001	0.001

M: Selective average, m: Error of the mean; ΔM: Absolute measurement error with a reliable probability of p > 0.95 (that is, the measurement result X falls within the interval X = M ± ΔM); s2: Average sample variance.

Table 3. Correlation matrix between morphometric indicators of eggs in the research group of Rhode Island laying hens

Indexes	Egg mass	Egg shell thickness in the middle part	Egg shell thickness at the sharp end	Egg shell thickness at the blunt end
Egg mass	1			
The thickness of the egg shell in the middle part	0.916	1		
The thickness of the egg shell at the sharp end	0.886	0.992	1	
The thickness of the egg shell at the blunt end	0.889	0.979	0.967	1

Table 4. Correlation matrix between morphometric indicators of eggs in the control group of Rhode Island laying hens

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Indexes	Egg mass	Egg shell thickness in the middle part	Egg shell thickness at the sharp end	Egg shell thickness at the blunt end
Egg mass	1			
The thickness of the egg shell in the middle part	0.750	1		
The thickness of the egg shell at the sharp end	0.762	0.950	1	
The thickness of the egg shell at the blunt end	0.750	0.999	0.950	1

When examining these correlation matrices, a reliable correlation was established between the indicators of egg mass and shell thickness at the level of very high and high correlations in the experimental (0.886–0.916) and control (0.750-0.762) groups. This finding underscores the close interdependence between these variables in the studied groups.

The following macromorphological changes were revealed during the external examination of the eggs. A softened (insufficiently mineralized) shell was found in some of the eggs, which could often be destroyed when the eggs were collected from the nest (Figures 1, 2). The eggs of chickens in the experimental group indicated damage to the shell in the area of the blunt end-small cracks in the form of spider-like branches and bumpy thickenings on its surface (Figure 2). Some eggs laid by chickens in the experimental group retained an intact shell, characterized by small, fragile layers of white color on the outer surface, as depicted in Figure 3. The hens in the experimental group displayed noticeable signs of defects in the eggshell texture. These included complete or partial depigmentation, the emergence of roughness marked by spilled thickenings, and calcareous nodules across the entire surface. Additionally, local contamination of the shell surface with blood was observed (Figure 4). Hens of the control group laid conditioned eggs, which had a characteristic monoasymmetric shape with an apical narrowed end and a blunt lower end, as well as a solid, smooth, clean shell, uniformly colored in a light beige color (Figure 5). Pigmented and depigmented spots, as well as deformations in the form of bumps or areas of thickening and depressions, mainly in the area of the blunt end, were found on the egg shell of the chickens in the experimental group (Figure 6). In 80% of eggs from hens in the experimental group, contamination of the shell with traces of feces and/or blood was observed, particularly in combination with its roughness and pigmented speckled spots (Figure 7). Some of the chickens in the experimental group laid eggs without calcified shells. Such eggs were easily broken, so when inspecting the territory of the poultry farm, only their fragments were often found, in particular, the remains of the inner shell. There was also a rupture of this shell in the middle part (Figure 8). Some of the eggs of the chickens in the experimental group had significant deformations. Their shells were weakly calcified and poorly designed, with violations of the usual egg shape, with layers of white granular substrate on their outer surface and partial minor contamination with blood (Figure 9). When examining the internal content of the eggs of the experimental group, bloody spots were found in the protein part in 12% of cases. Compared with the indicators of the internal content of eggs of hens of the control group, the yolk of the eggs of the experimental group was smaller in size and lighter in color, and the protein part was rare (Figure 10). The specified defects of the shell and egg contents of the chickens of the experimental group became the basis for their culling as unsuitable for transportation and/or incubation.

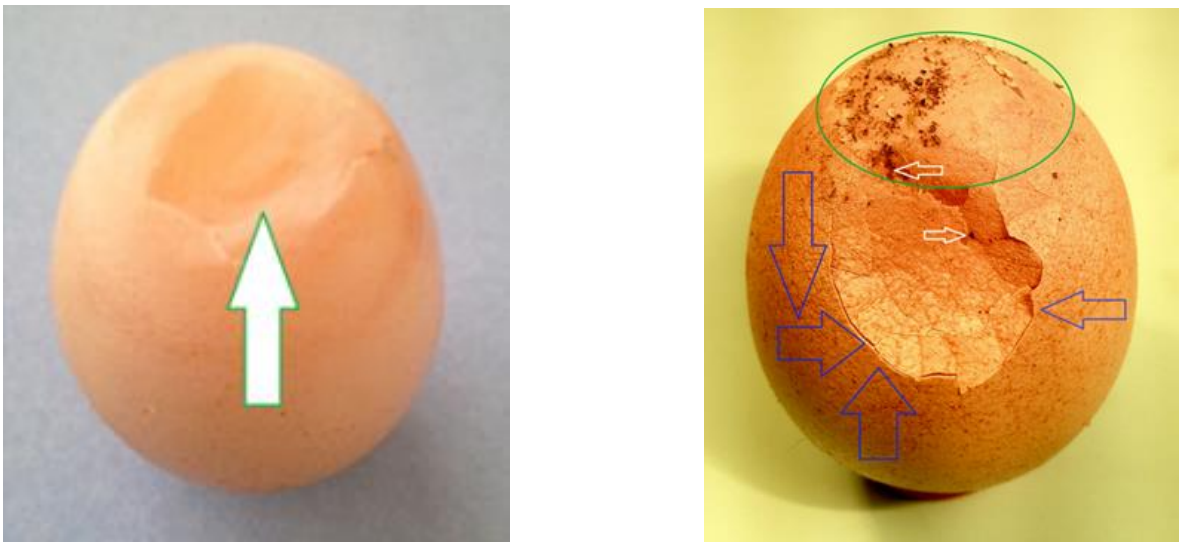

Figure 1. Cull eggs with a defect of insufficient calcification- a thin shell (arrow) with small tubercles on the outer surface (blue arrows), blood stains (white arrows) and contamination (oval) from a domestic hen (Rhode Island, age 1.5 years, experimental group).

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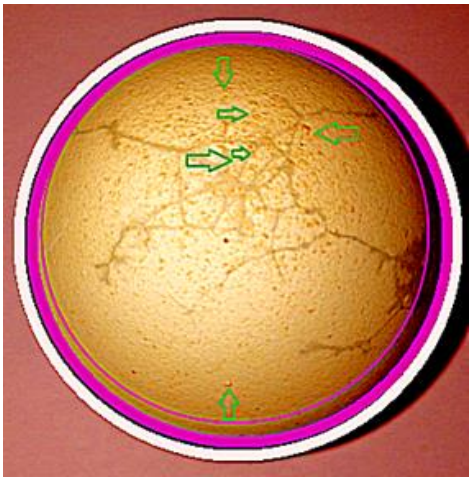


Figure 2. Substandard egg with combined shell defects (fine cracks and small bumpy thickenings–green arrows) in the blunt end region from a domestic hen (Rhode Island; age 1.5 years, experimental group).

Figure 3. The appearance of an egg with white brittle layers on the outer surface of the shell from a domestic chicken (Rhode Island breed; age 1.5 years, experimental group).

Figure 4. An unconditioned egg with a bumpy surface defect of a depigmented shell, unusually light-color (oval), and blood stains (arrow) from a domestic chicken (Rhode Island breed; age 1.5 years, experimental group).

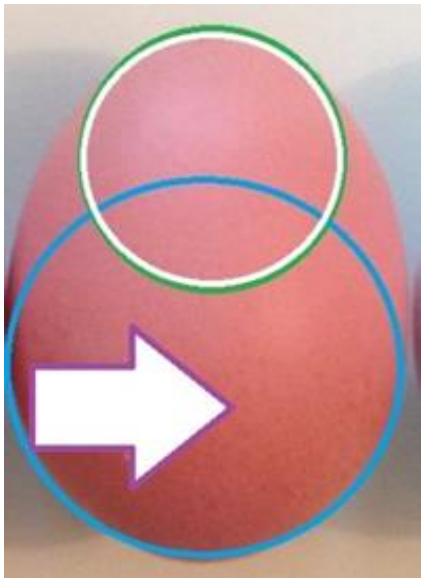
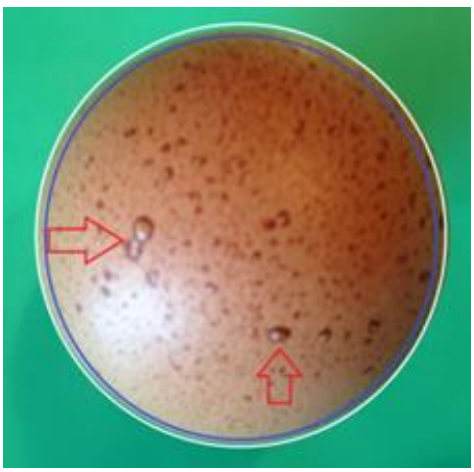


Figure 5. A conditioned egg of a preserved form from a hen (Rhode Island breed; 1.5 years old) of the control group: the surface of the shell is intact, smooth, clean, and uniformly colored in a light beige color (arrow).



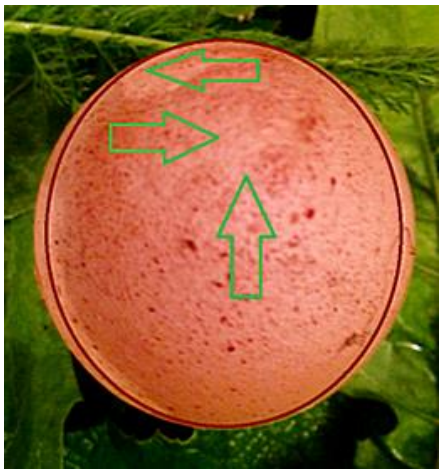


Figure 6. The surface of substandard eggs from the extended (blunt) end side with the presence of small pigmented and depigmented spots, diffuse thickenings and indentations on the surface of the shell (arrows in the oval) from a domestic chicken (Rhode Island, 1.5 years, experimental group).

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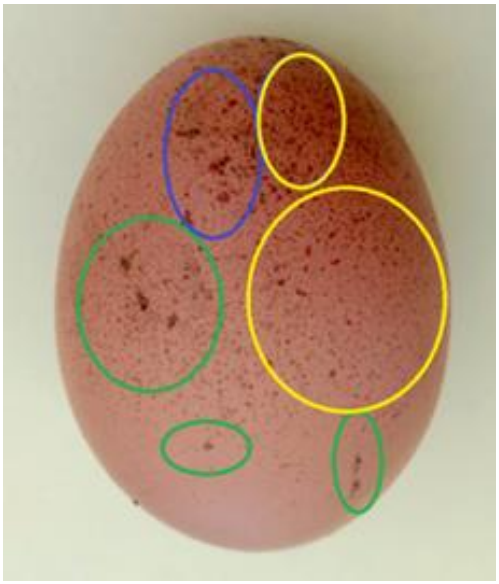

Figure 7. The surface of an unconditioned egg with the presence of pigmented spots (yellow ovals), contamination with blood (blue oval), and droppings (green ovals) in a domestic hen (Rhode Island breed, 1.5 years old, experimental group).

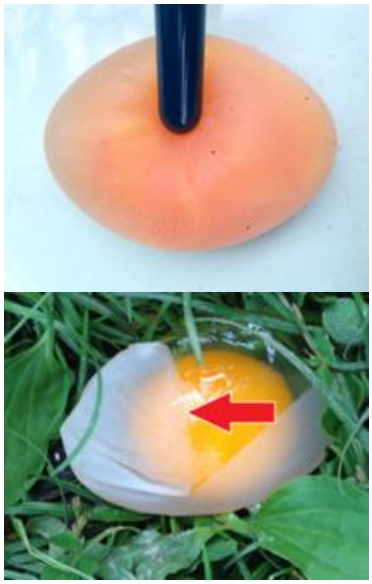

Figure 8. View of substandard eggs without calcified and torn shells in the middle part (arrow) laid on a pasture by a domestic hen (Rhode Island; 1.5 years old, experimental group).

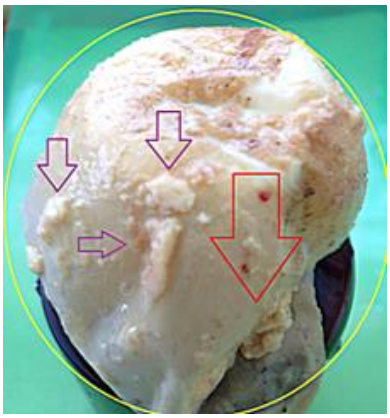

Figure 9. A fragment of a weakly calcified eggshell of a domestic chicken (Rhode Island breed; 1.5 years old) with the presence on the surface of layers of white color (purple arrows) with blood impurities (Two red spots in the red arrow).

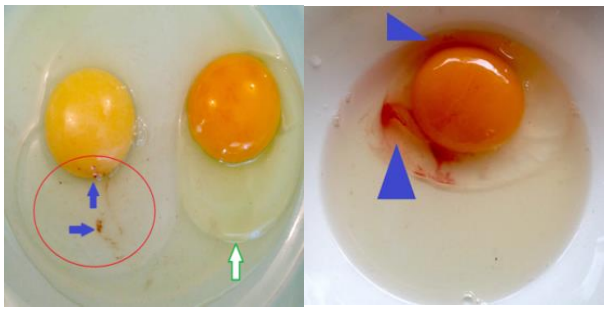

Figure 10. The internal content of eggs of domestic chickens (Rhode Island breed; 1.5 years old) of the experimental (left and right eggs) and control group (the egg in the center). The eggs of the experimental group have a rarefied protein, bloody content (blue arrows in a red oval), and a comparatively lighter yolk. An egg in the center of the control group with a bright yellow yolk and dense white (white arrow).

DISCUSSION

Considering the obtained results on the reduction of egg productivity in chickens with spontaneous *Eimeria-Histomonosis-Trichomonosis* co-infection, it should be noted that many poultry diseases are characterized by a decrease in egg production and/or egg mass. This is confirmed by research conducted on adult chickens and turkeys (Richter et al., 2010; Landman et al., 2016) regarding the negative impact on the body and threats to the health of the bird and, accordingly, the impact on its productivity (Saikia et al., 2023). The *Eimeria-Histomonosis-Trichomonosis* co-infection in the experimental group of chickens exhibited a chronic latent course without prominent clinical signs, consistent with observations by other researchers (Amin et al., 2011). As the intensity of co-infection, especially involving its components, such as *Trichomonas gallinarum* and *Histomonas meleagridis*, increases, there is a heightened risk of invasion outbreaks (Amin et al., 2014). These outbreaks can lead to severe consequences, including high mortality, which was confirmed by Feng et al. (2021) for the study populations of certain species of wild birds. It is known that birds often suffer from eimeriosis, histomoniasis, and trichomoniasis (Tuska-Szalay et al., 2022). Co-infection undeniably exerts a negative impact on the health of laying hens, with a particular emphasis on production rates, ultimately leading to a decrease in egg production (McDougald, 2005; Hess et al., 2015). In the present study, egg production was reduced by 52%, and egg mass decreased by 19.37% during eimeriosis, Histomonasis, and trichomoniasis co-infestation in laying hens. These findings align with the observations reported by Amin et al. (2011)

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and Landman et al. (2021), particularly in the context of trichomoniasis in hens. Both studies noted decreased egg production and a loss of average egg weight without any clinical signs. Indicators of reduced egg production and/or loss of average egg mass in hens, even in the absence of apparent disease symptoms, could serve as a basis for suspecting co-infection and require a comprehensive, including parasitological, investigation.

Together with a decrease in the level of egg production (gross collection of eggs) and egg weight in infested hens, the changes in the macromorphological indicators of eggs were determined. Their manifestation was influenced by the combined destructive action of pathogens of *Eimeria-Histomonosis-Trichomonosis* co-infection in relation to various structural targets of the chickens' bodies. *Eimeria acervulina* (Tyzzer, 1929), *Eimeria brunetti* (Levine, 1939), *Eimeria maxima* (Tyzzer, 1929), *Eimeria mitis* (Tyzzer, 1929), *Eimeria necatrix* (Jonson, 1927), *Eimeria praecox* (Jonson, 1927), and *Eimeria tenella* (Raillet and Lucet, 1891) caused the development of pathological processes in the intestinal canal, which were characterized by damage to the epithelial cells of the mucous membrane of the wall of the intestinal tube along its entire length (Dalloul and Lillehoj, 2006). Damage to the intestinal canal is caused by the pathogens *Histomonas meleagridis* and *Trichomonas gallinarum*, negatively affecting its terminal part (Lee, 1972) and causing granulomatous damage (Landman et al., 2019) to internal organs (Liebhart et al., 2014; Fadhil et al., 2020). Researchers reported a decrease in egg production, egg weight, shell condition, and changes in the internal content of eggs in birds with trichomoniasis and associate this with the development of degenerative changes in its genital organs due to liver damage (Narcisi et al., 1991). This finding is also consistent with the results of studies by Fitz-Coy and Edgar (1992).

In chickens with *Eimeria-Histomonosis-Trichomonosis* co-infection, there was a high probability of a decrease in the absorptive function of the intestinal mucosa. This refers to insufficient intestinal absorption of various substances in infested chickens, especially those involved in forming the egg and its shell (Dalloul and Lillehoj, 2006). Researchers (da Costa Freitas, 2014) experimentally proved the occurrence of changes in the structure of intestinal villi during eimeriosis in chickens, which prevented the absorption of nutrients, such as calcium, phosphorus, magnesium, proteins, and lipids, leading to decreased weight and productive qualities of chickens. The deficiency in calcium ions affects the quality of the eggshell (Yan et al., 2016; Oikeh et al., 2019). Egg shell formation largely depends on Vitamin D content (Babazadeh et al., 2022). Partly due to the mentioned reasons, the eggs of the chickens of the experimental group showed signs of shell formation disorders, which, according to the morphological evaluation of the egg, was manifested by the presence of its textural defects (softening/insufficient mineralization), cracks and bumpy thickening on the shell. As a result of calcium deficiency, infested hens laid eggs with insufficiently formed, softened shells or without them (Yan et al., 2016; Oikeh et al., 2019).

Contamination of the eggshells of experimental chickens with droppings indicated the presence of inflammatory processes in their intestinal tract, and it can result in diarrhea. Slight contamination of the eggshell surface with blood indicated the presence of probable inflammatory processes in the egg-forming organs. It is important that the egg yolks of chickens with spontaneous *Eimeria-Histomonosis-Trichomonosis* co-infection have a lighter color, compared to the yolks of eggs of healthy chickens. The intensity of the color of the egg yolk depends on the level of carotenoids. This is confirmed by the report of Ruff and Fuller (1975), who reported a decrease in the absorption of carotenoids, resulting in light pigmentation of egg yolks during eimeriosis in chickens. The risk of such a phenomenon naturally increases when the intestines of chickens are affected by *Eimeria*, *Histomonas*, and *Trichomonas*. The rarefaction of protein in the eggs of co-infested chickens in the current study is consistent with previous studies (Teng et al., 2021; Kim et al., 2022) and is logically explained by a general protein deficiency due to a decrease in the intestinal absorption of proteins, amino acids and other nutrients protein (Vakili et al., 2021).

CONCLUSION

According to the results of the current research, spontaneous *Eimeria-Histomonas-Trichomonas* co-infection had a negative effect on the productive qualities of laying hens. The findings indicated a decrease in the egg-laying rate, egg mass, a significant decrease in shell thickness, and manifestations of macromorphological changes in eggs (defects in the shell and internal contents). The specified macromorphological changes and egg defects result from the negative impact of co-infection on oogenesis and the processes of egg formation and indicate the systemic nature of the lesions and the morphofunctional insufficiency of the egg-forming organs. The prospect of further research requires studying the state of the reproductive organs of domestic laying hens during co-infestation.

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Authors' contribution

Petro Liulin conducted coproscopic studies and described his results, and together with the owners of the farm, he formed experimental and control groups of chickens. Lyubov Lyakhovych conducted macromorphological studies of the eggs of chickens of the experimental and control groups, took photographs, and analyzed the obtained data. Mykola Bogach performed the determination of the species relationship of co-infection pathogens. Alla Petrenko participated in the determination of egg productivity of chickens and morphometric indicators of eggs. Inna Kostyuk took part in the detection of deformations of eggs. Petro Lyulin, Lyubov Lyakhovych and Mykola Bogach conducted the literature analysis and wrote the manuscript. All authors have reviewed and approved the final version of the manuscript for publication in this journal.

Competing interests

The authors declare no conflict of interest.

Ethical considerations

All authors reviewed the manuscript for ethical issues such as plagiarism, consent to publish, misconduct, forgery and/or falsification of data, re-publication and/or submission, and redundancy.

Availability of data and materials

Authors of this article confirm the availability of data and materials of valid research and availability upon reasonable requestS.

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