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Macro- and microstructure of the oesophageal tonsil in turkeys during the post-vaccination period

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Abstract. The successful development of poultry farming depends on the state of the immune system of birds, which from the first days of life come into contact with a significant amount of antigens that enter the body mainly through the digestive tract and provoke the development of diseases. Food retention in the transition zone between the oesophagus and stomach leads to the development of the oesophageal tonsil, as one of the most developed immune formations. The purpose of this study was to find out the development of the oesophageal tonsil in turkeys and determine the timing of its full morphofunctional maturity by vaccine prevention. Material for macro- and microscopic studies was selected from 66 individuals of Big-6 turkeys in the early stages of the postnatal period of ontogenesis, which were divided into experimental and control groups. When performing the study, classic morphological research methods were used. It was shown that in both groups of turkeys, the levels of structural organisation of the lymphoid tissue that forms the base of the oesophageal tonsil arise in a certain sequence, but with different intensity. In day-old birds, the first level was revealed – diffuse lymphoid tissue, represented by local clusters of diffusely located lymphocytes, some of which migrate into the surface epithelium and have close contact with epithelial cells. On day 10, the experimental group of turkeys developed pre-nodes with dense arrangement of lymphocytes without a capsule (second level) and primary lymphoid nodules with

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compact arrangement of lymphoid cells, limited by a capsule (third level), and on day 20, secondary lymphoid nodules with light centres and a well-defined mantle zone (fourth level) appeared, while in the control group, pre-nodes were recorded at 10 days of age, and primary and secondary lymphoid nodules at 20 days of age. This may indicate that on day 20, the oesophageal tonsil, as an immune formation, acquires morphofunctional maturity, and its cells are able to recognise and destroy specific antigens. The results obtained contribute to elucidating the natural mechanisms of development of immunological processes in poultry in ontogenesis, which should be considered by veterinarians when developing new vaccine prevention strategies

Keywords: junction of the oesophagus and stomach; lymphoid tissue; lymphoid nodules; morphological studies; lymphocytes; poultry

Introduction

The digestive organs of poultry are constantly exposed to various negative environmental factors that come with food and water. On their penetration, specific cellular reactions of the immune system occur, which neutralise foreign genetic information and ensure the development of cellular and humoural immunity. The development of an immune response largely depends on immunoprophylaxis against infectious diseases of poultry.

Along with obtaining highly productive poultry, according to T.S. Budnik & S.V. Guralska (2023), there is a need to ensure its sustainable well-being, a more in-depth knowledge of the mechanisms of natural immunological processes, and the impact of vaccination on them. Unlike mammals, as found by J.-C. Weil *et al.* (2023) and R. Capotă *et al.* (2025), the organs of the immune system of birds are characterised by weak development of lymph nodes (ducks, geese, swans) or their absence (chickens, turkeys, quails), the absence of a pharyngeal lymphoid ring and the presence of a cloacal sac, Gardner's gland, and well-developed lymphoid tissue that is associated with mucous membranes (mucosa associated lymphoid tissue – MALT) and as a morphological substrate forms the basis of specialised structures – immune or lymphoid formations. The latter are peripheral organs of the immune system and are always located on the path of possible penetration of genetically foreign substances into the body and participate in the mechanisms of immune defence. One of the first powerful protective barriers of the digestive canal, as reported by G. Garagulya *et al.* (2022), is the oesophageal tonsil, which is located in the thoracic-abdominal cavity at the junction of the oesophagus with the glandular part of the stomach. Its development in birds of different species depends on age, sex, conditions of keeping and feeding, physiological characteristics of the body, and the activity and duration of action of feed and water antigens on the junction. In addition, within the same age group, there are always slight fluctuations between the maximum and minimum numbers, content, size, and other parameters of the structural elements of the oesophageal tonsil, which serve as a manifestation of the body's immune defence.

As noted by S. Ceccopieri & J.P. Madej (2024), the lymphoid tissue of the peripheral organs of the immune system, including the oesophageal tonsil, is quite early exposed to various antigenic substances, under the influence of which T- and B-lymphocytes acquire specific immunoreactivity and provide a full-fledged immune response. In this regard, T. Etekhari *et al.* (2022)

highlighted four stages of differentiation of lymphoid tissue. The first stage can be considered the appearance of diffuse ("associated") lymphoid tissue in the mucous membrane of the digestive canal organs, which is represented by lymphocytes forming several rows of cells, plasmocytes and macrophages. The presence of lymphoid cells in the mucous membrane is considered as the body's readiness to meet, recognise, and neutralise foreign substances (antigens) that are located in the external environment (digestive canal). The second stage is the development of separate clusters of lymphoid cells near blood vessels and in the thickness of exocrine glands, etc. Such structures are the pre-nodular stage of development of peripheral organs of the immune system. The next, third stage in the development of lymphoid tissue is the appearance of primary lymphoid nodules, which are represented by denser clusters of cells of the lymphoid series of rounded and oval shapes and clear contours. Their presence can be considered as a state of high morphological maturity of the immune system organs, that is, their readiness to form reproduction centres for local production of lymphoid cells. The last, fourth stage of development of lymphoid tissue, which is the highest degree of differentiation of the immune system organs, is considered to be the appearance of reproduction centres (germinal, light) in the nodules. Such centres, as noted by M. Bemark *et al.* (2024) and N.J. Monisha *et al.* (2024), occur due to long-term active strong antigenic effects, when immunological activity appears and, accordingly, the need to increase the population of lymphoid elements. For the peripheral organs of the immune system, as found by G. Garagulya *et al.* (2022), is characterised by early involution, which begins with the onset of sexual maturity and is manifested by a gradual decrease in the area of lymphoid tissue, which significantly reduces the number of lymphoid nodules with the growth (replacement) in its place of loose fibrous connective and adipose tissues.

The features of the microstructure and cellular composition of the lymphoid tissue of the digestive canal are relatively well studied in chickens, ducks, and individual wild birds with different types of nutrition and digestive activity, as noted by N.D. Rahman & S.K. Waad (2025). Information on the morphological aspects of the development of the oesophageal tonsil in turkeys is scarce and insufficiently clarified, and there is no information on the effect of vaccination on its development and the onset of its morphofunctional maturity. In this regard, the purpose of the current study was to investigate the development and morphofunctional structure of the oesophageal tonsil in vaccinated and unvaccinated turkeys, which is important for establishing their immunological status in different age periods and evaluating the effectiveness of veterinary and preventive measures in poultry farms.

Literature Review

T.S. Budnik & S.V. Guralska (2023) noted that the epizootic well-being of poultry complexes largely depends on the state of the bird's immune system and provides for immunoprophylactic measures against infectious and bacterial diseases. Specialised lymphocytes, plasma cells, and macrophages, which are part of the lymphoid tissue associated with the mucous membrane of the digestive canal, participate in immune responses. V. Khomich *et al.* (2020) based on morphological features, classified the lymphoid tissue of the digestive canal of birds into structured (tonsils, single and aggregated lymphoid nodules) and diffuse, including cellular elements (intraepithelial lymphocytes, macrophages, plasma cells, lamina propria lymphocytes).

L.O. Bugay (2008) noted that macroscopically, the oesophageal tonsil is visible in musk

ducks at 5 and 10 days in the form of indistinct spots, and from 15 days it acquired a rectangular shape with multiple elevations of a rounded shape, which, according to the researcher, indicates a significant localisation in these areas of lymphoid tissue. Its most intensive growth was noted during the periods from 15 to 30 days and from 90 to 180 days, simultaneously, the indicator of maximum length was recorded in ducks on day 150, and width – in 120-day-old birds. V.R. Indu *et al.* (2020) conducted histomorphological studies of the oesophageal tonsil of broiler ducks aged 6 to 8 weeks. They found six to eight isolated tonsils near the base of mucosal folds that did not form a continuous ring and consisted of many large lymphoid nodules separated by internode sections. According to H.H. Dönmez *et al*. (2012), a well-developed oesophageal tonsil compensates for the absence of palatine tonsils in birds. The secretory sacs of the ducks' oesophageal glands were in cooperation with lymphoid tissue, and the simple cylindrical epithelium turned into lymphoepithelium, as reported by V.T. Khomych & S. Usenko (2013). The researchers noted that the composition of lymphoid tissue includes small, medium, and large lymphocytes, plasma cells and macrophages, and the presence of venules with high endothelium, in their opinion, indicates a close immunological connection of the oesophageal tonsil with other organs of the lymphatic system.

According to N. Nagy *et al.* (2005), the oesophageal tonsil of chickens is anatomically located proximal to the stomach, which allows it to be exposed to antigenic environmental influences. It contains about eight isolated units (accumulations of lymphoid tissue) at the base of each fold of the oesophagus, and therefore, their number corresponds to the number of oesophageal folds. Each isolated unit contained crypts bounded by the lymphoepithelium and adjacent lymphoid tissue. A similar number of isolated units in the oesophageal tonsil of 12-week-old White Leghorn chickens was observed by V.R. Indu & K.M. Lucy (2021). The researchers noted a close relationship between the epithelium of the oesophageal glands and lymphoid tissue.

V.T. Khomich *et al.* (2020) conducted morphological studies of the oesophageal tonsil of sexually mature birds of various species in a comparative aspect. They noted that chicken, duck, goose, quail, turkey, guinea fowl, pheasant, grouse, peacock, pheasants, black goose, stork, magpie, and crow, it was visible as a thickened whitish-pink stripe that covers the perimeter (compact tonsils), and in pigeon, waterhen, partridge, jay, and coot, immune formation was not visible (diffuse tonsils). The maximum values of oesophageal tonsil length were recorded in the black goose (47.5 ± 0.44 mm) and stork (46.23 ± 0.37 mm), and the smallest – in quail (9.26 ± 0.09 mm) and magpie (8.19 ± 0.13 mm). Its largest width was inherent in the duck (9.25 ± 0.08 mm), and the smallest – magpie (1.16 ± 0.06 mm) and crow (1.04 ± 0.06 mm). The largest area of the mucous membrane with lymphoid tissue was occupied in the duck (68.64 ± 0.70%), and the smallest – in the partridge (2.31 ± 0.05%). Its individual structural elements were also found in the muscular and serous membranes of birds of certain species. According to the researchers, the unique anatomical location of the oesophageal tonsil is important for the development of cellular and humoural links of the immune response after oral administration of vaccines.

Materials and Methods

Macro- and microscopic studies of the oesophageal tonsil of turkeys were conducted during 2024 and until July 2025. The resource and facilities were provided by the certified educational, scientific and production laboratory "Centre for Biomorphological Technologies" of the

Department of Vertebrate Biomorphology named after Academician V.G. Kasyanenko of the National University of Life and Environmental Sciences of Ukraine. Research was conducted in compliance with the principles of the European Convention for the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes (1986), ARRIVE recommendations (n.d.) on reporting on experiments with live animals, and Law of Ukraine No. 3447-IV (2006). To euthanise the birds, in accordance with Order of the State Committee of Veterinary Medicine of Ukraine No. 365 (2010), barbiturate anaesthesia was used, namely sodium thiopental at a dosage of 7-20 mg/kg (depending on body weight) by intravenous injection. The decision of the bioethical Commission of the National University of Life and Environmental Sciences of Ukraine of Ukraine on granting consent to the use of animals for scientific purposes (No. 045/2025 of June 26, 2025) was received to conduct research.

Material for the study was selected from 66 female clinically healthy turkeys of the hybrid broiler breed Big-6 in six age groups: 1, 10, 20, 30, 40, and 50 days of age. The day-old birds were divided into experimental (vaccinated) and control (unvaccinated) groups. The experimental group received vaccines in accordance with the scheme of vaccine prevention and therapeutic treatments of the poultry farm LLC "Volodar" in the Bila Tserkva district of the Kyiv Oblast. Turkeys were vaccinated against Newcastle disease and Marek's disease immediately after hatching; against coccidiosis at one day old; against viral rhinotracheitis at 7, 21, and 42 days old; at 14 days old, they were revaccinated against Newcastle disease; and vaccinated against haemorrhagic enteritis at 28 days old. Birds of the second group of preventive vaccinations were not used. Turkeys of both groups were kept in a poultry farm. According to the diet, young animals were fed starter compound feed with a high protein content (up to 28%), and over time it was replaced with mixed feed containing a lower proportion of protein and more grain mixture for more intensive development of poultry.

Macroscopic, microscopic, morphometric, and statistical methods of studying the oesophageal tonsil of turkeys of the experimental and control groups were used to perform these tasks. The initial (anatomical) level of the study was carried out, which provided for the slaughter and exsanguination of the bird, its opening and preparation of the oesophageal tonsil area with its extraction for further morphological studies. Macroscopic studies determined the features of localisation, colour, shape, and linear parameters of the length and width of the oesophageal tonsil of turkeys in the age aspect using a calliper and ruler, followed by sampling for microscopic studies. The samples were placed in histological cassettes and immersed in a 10% aqueous neutral formalin solution for 48 hours. They were then washed for 24 hours in tap water. To remove water from the samples, dehydration with ethyl alcohol of increasing concentration (60°, 70°, 96°, and 100°) was performed for one to three hours. In the future, the material was passed through a mixture of alcohol and chloroform, then kept in pure chloroform, after which the material was placed in a mixture of chloroform-paraffin (at a temperature of 37°C) in and in paraffin (at a temperature of 55-56°C). A stainless steel filling and moulding station was used to produce the paraffin blocks.

Histological sections up to 5-10 microns thick were made from the obtained paraffin blocks using an MPS-2 sled microtome (Med-Tech-Price, Ukraine). Subsequently, histological sections were stained with haematoxylin and eosin according to Mallory and impregnated according to Kelemen using standard methods. Calculation of the area of lymphoid tissue and its structural and functional levels was carried out by the "point counting"

method using a binocular microscope and a measuring grid. The size of round and oval lymphoid nodules was determined using Olympus CX 43 microscope (Labdepo, Japan) and a micrometre eyepiece.

The results obtained during the research were recorded in protocols. Statistical processing of the results (mean, standard deviation) was performed on a personal computer using StatSoft Statistica 13.1 (2016) software suite. The assessment of statistical reliability was determined by the Student's t-test at three probability levels $P < 0.05$, $P < 0.01$, and $P < 0.001$. Micrographs of individual histoses of the location of the oesophageal tonsil of turkeys were performed using a microscope equipped with a Primo Star digital camera (Carl Zeiss, Germany) and connected to a personal computer.

Results and Discussion

Features of the macroscopic structure of the oesophageal tonsil

As the results of studies showed, in the area of the oesophageal tonsil, the mucous membrane formed low, wide longitudinal folds (6-8) and was more thickened compared to the cervical part of the oesophagus. V.T. Khomich *et al.* (2020) noted that the domestic goose and the Canadian black goose at the border of the oesophagus and stomach do not have the folding of the mucous membrane and cone-shaped papillae of the excretory ducts of the glands characteristic of the glandular part of the stomach. According to the researchers, such a transition zone is anatomical and physiological feature that is characteristic of this bird species and is associated with their trophic specialisation.

Macroscopically, the oesophageal tonsil was viewed on the inner surface of the wall of the transition of the oesophagus to the stomach of turkeys of the experimental group, starting from the 10-day age (Fig. 1A), and the control group – from the 20-day age. It had the appearance of a thin whitish ring-shaped strip with a bumpy surface and holes of crypts, which was located around the perimeter of the site at the base of the folds of the mucous membrane and between them. In older birds, the folds became more visible visually, and the colour of the tonsil changed to pinkish-whitish with a yellowish tinge (Fig. 1B). This is consistent with data by V.T. Khomych & S.I. Usenko (2013), who, when studying the oesophageal tonsil in ducks, noted that the corresponding colour and bumpy surface of such an immune formation are associated with the localisation of significant accumulations of lymphoid tissue in it.

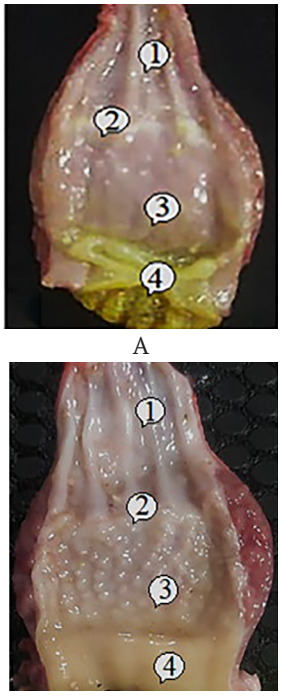


Figure 1. Oesophageal tonsil in turkeys of the experimental group aged 10 (A) and 30 (B) days

***Note**: 1 – oesophagus; 2 – area of localisation of the oesophageal tonsil; 3 – glandular part of the stomach; 4 – muscular part of the stomach with cuticle (A) and without cuticle (B). Native drugs*

***Source**: photo by the authors*

With increasing age of turkeys, macroscopic indicators of the length and width of the oesophageal tonsil increased (Table 1). In the poultry of the experimental group, the tendency to increase these indicators was observed from the 10-day age, and the control group – from the 20-day age. The length and width of the oesophageal tonsil reached maximum values in a 50-day-old bird. In addition, they were slightly larger in the group of turkeys that were vaccinated.

Table 1. Macroscopic parameters of the oesophageal tonsil in turkeys, mm, M±m, n=6

Poultry group	Length	Width
	Day 10	
Experimental	11.5 ± 0.28	2.08 ± 0.15
Control	-	-
	Day 20	
Experimental	16.80 ± 0.15	3.80 ± 0.21
Control	15.22 ± 0.48	3.16 ± 0.15
	Day 30	
Experimental	23.22 ± 0.34	4.87 ± 0.08***
Control	21.37 ± 0.23	4.50 ± 0.28*
	Day 40	
Experimental	26.72 ± 0.32	6.03 ± 0.21*
Control	23.76 ± 0.14*	5.10 ± 0.07**
	Day 50	
Experimental	28.75 ± 0.17*	6.80 ± 0.15**
Control	27.28 ± 0.19	5.92 ± 0.13**

***Note**: Macroscopic parameters of the oesophageal tonsil in turkeys, mm, M ± m, n = 6*
***Source**: developed by the authors*

On average, in birds of the experimental group from 10 to 50 days, linear indicators of the length and width of the oesophageal tonsil increased by 150.0% (2.5 times) and 226.92% (3.2 times), respectively. In the comparative aspect of turkeys in both groups, an increase in these indicators was observed from 20 to 50 days of age. Thus, the length and width of the oesophageal tonsil in the experimental group increased by 71.13% (1.71 times) and 78.95 % (1.78 times) during this period, and in the control group – by 79.24% (1.8 times) and 87.34% (1.9 times), respectively. The growth of these indicators was uneven. The length and width of the oesophageal tonsil in turkeys of both groups increased most intensively from 20 to 30 days: in the experimental group, respectively, by 38.21% and 28.16% ($P < 0.001$), and the control group – by 40.41% and 42.41% ($P < 0.05$). With a lower intensity, these indicators increased in poultry of the experimental group from 40 to 50 days (by 7.59%, $P < 0.05$ and 12.77%, $P < 0.01$, respectively) and the control group from 30 to 40 days (by 11.18%, $P < 0.05$ and 13.33%, $P < 0.01$, respectively). Thus, macroscopically, from day 10, the oesophageal tonsil in turkeys of the experimental group was detected in the form of a whitish stripe and had slightly higher indicators of length and width, compared to the tonsil in the control group, which was observed starting from the age of 20 days. The most intense increase in these indicators in birds of both groups was observed at 3-4 weeks of their development.

Histotopography and microstructure of the oesophageal tonsil

The microscopic structure of the oesophageal tonsil in turkeys of both groups is similar to the thoracic-abdominal part of the oesophagus, but it is somewhat thickened. The wall of this area was formed by three membranes: mucous, muscular, and serous (Fig. 2). The mucous membrane had a characteristic structural relief, the elements of which were longitudinal folds separating crypt-like formations, the development of which is conditioned by the deepening of the surface epithelium into its own plate. According to research data by A.A. El-Mansi *et al*. (2025), the number of folds in birds depends on their species characteristics, and the size of the folds in the area of the oesophageal tonsil is larger than in other parts of the oesophagus, which indicates the presence of lymphoid tissue in them.

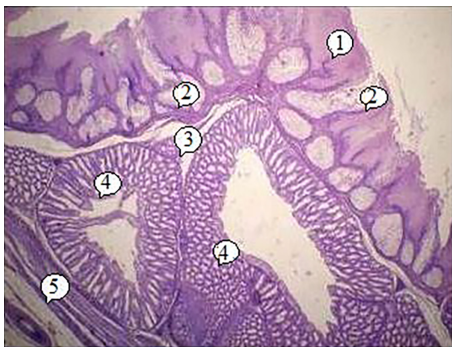


Figure 2. Area of transition of the oesophagus to the glandular part of the stomach in a day-old turkey

***Note**: 1 – oesophageal epithelium; 2 – oesophageal glands; 3 – submucosal base; 4 – lobules of deep glands of the glandular part of the stomach; 5 – muscle membrane. Staining with haematoxylin and eosin ×40*

***Source**: developed by the authors*

The epithelial layer of the mucous membrane passed from a multilayer flat slightly keratinised to a single-layer prismatic glandular one. The multi-layered epithelium is characteristic of the oesophagus and consisted of epithelial cells of the basal, spinous, or spiny and superficial layers. The cells of the basal layer are arranged in a single row and had a cylindrical shape with nuclei pronounced in the area of the basal pole. The spiny layer is represented by many rows of polygonal epithelial cells with processes, and the cells of the surface layer were placed in 2-5 rows. The latter had a pronounced flat shape, and destroyed nuclei were recorded in individual cells. The single-layer prismatic epithelium is characteristic of the glandular part of the stomach and is represented by cylindrical epithelial cells, which are arranged in a single row. Their surface was covered with mucus. In its own plate of the mucous membrane, the glands characteristic of the oesophagus were preserved, and the muscle plate, which was formed by bundles of smooth muscle cells, was intermittent. Lobules of deep glands, which are characteristic of the glandular part of the stomach, were observed in the submucosal base.

The muscle membrane of the oesophageal tonsil site was formed by undisturbed smooth muscle tissue that was adjacent to the submucosal base. Bundles of its cells formed the inner and outer longitudinal and middle – circular layers, which had different development. The circular layer was best developed, the inner longitudinal layer was slightly smaller, and the outer longitudinal layer was weaker. Between the muscle layers were small layers of loose fibrous connective tissue with pronounced blood vessels, nerve nodes and fibres. Outside, the area of the oesophageal tonsil of turkeys is covered with a serous membrane, which passed to the glandular part of the stomach. It was formed by a single-layer flat epithelium and loose fibrous connective tissue with a small number of blood vessels.

According to the conclusions of N. Hamoda & A. Farag (2018), changes in the epithelium from multi-layered to single-layered, and feed retention in the area of the oesophageal tonsil

leads to increased antigenic effects, and the development of lymphoid tissue in this area. A.R.H. Al-Fartwsy *et al.* (2025) reported that in guinea fowl, the muscle membrane of the oesophageal tonsil location is formed by two layers: circular (inner) and longitudinal (outer). According to research data by N. Dyshliuk *et al.* (2024) in the common blackbird, the inner layer of the muscular membrane was buried in separate large folds and was involved in their formation, and in the grey heron, this membrane was somewhat thickened and had significant vascularisation and innervation, which, according to L.P. Kharchenko *et al*. (2001), indicates the presence of a sphincter between the oesophagus and stomach.

The process of morphofunctional differentiation and specialisation of lymphoid tissue in the oesophageal tonsil of turkeys of both groups arose in the following sequence: at the beginning, diffuse clusters of lymphocytes placed in their own plate of the mucous membrane appeared, then – pre-nodules, primary lymphoid nodules were formed from the latter, and secondary lymphoid nodules with light centres developed on their base due to the action of antigens. Such a full range of levels of structural organisation of lymphoid tissue, according to I. Oláh *et al.* (2014), may indicate the functional maturity of the oesophageal tonsil in birds, i.e., its ability to destroy and remove the antigen recognised by lymphocytes.

At day-old age, the lymphoid tissue in the mucous membrane of the oesophageal tonsil area was represented only by separate, structurally homogeneous clusters of diffusely located lymphocytes, without noticeable rarefactions or compactions in the centre (Fig. 3). Foci of diffuse lymphoid tissue were located mainly under the surface epithelium in its own plate of the mucous membrane. Some of the lymphocytes migrated to its lower layers and established a "lymphoepithelium", which had tight contacts of lymphoid and epithelial cells. Individual lymphocytes were in a mitotic state.

Figure 3. Diffuse lymphoid tissue at the junction of the oesophagus to the glandular part of the stomach in a day-old turkey
***Note**: histological preparation: 1 – mucous membrane; 2 – oesophageal epithelium; 3 – oesophageal glands; 4 – diffuse lymphoid tissue; 5 – lobule of the deep gland of the glandular part of the stomach; 6 – muscle membrane. Staining with haematoxylin and eosin ×40*
***Source**: developed by the authors*

During lymphocyte infiltration, the epithelium was locally disturbed and became spongy. Minor accumulations of diffuse lymphoid tissue were also recorded near blood vessels, with the terminal parts of the oesophageal glands and their ducts, and near the lobules of deep glands that belong to the glandular part of the stomach. In addition to elastic and collagen fibres, reticular cells were recorded in the areas of lymphocyte clusters. The latter had different orientations and, together with reticulocytes, formed a structure similar to a "grid", in the loops of which there were phagocytic macrophages and lymphoid cells of varying degrees of maturity.

According to I. Oláh *et al.* (2014), among lymphocytes migrating to the epithelium, small forms predominated, which were characterised by a high nuclear-cytoplasmic ratio. According to the researchers, such movement and accumulation of lymphocytes towards the lumen (external environment), which is enriched with

microflora with its diverse antigenic spectrum, indicates that lymphocytes are immunocompetent cells that maintain the constancy of the internal environment and protect against infections. As noted by L.V. Chernyshenko & S.T. Chernokulskiy (1986), the location of lymphoid tissue along the course of the lymph and haemomicrocirculatory bed is a pattern and indicates close contact of the immune system organs with the microcirculation system and blood enrichment throughout their entire length with lymphocytes.

At 10-day age, the area of localisation of lymphoid tissue in the oesophageal tonsil mucosa increased by 82.05% (1.8 times) in turkeys of the experimental group and by 46.38% (1.4 times) in the control group compared to this indicator in day-old poultry (Table 2). Accumulations of diffuse lymphoid tissue were found in the area of the base and apex of the folds of its own plate and partially submucosal base of the mucous membrane. The area of lymphoid infiltration of the epithelial layer, oesophageal gland packets and their excretory ducts also increased.

Table 2. Area of the mucous membrane in the area of the oesophageal tonsil in turkeys, %, M ± m, n = 6

Poultry group	Mucosal area	
	without lymphoid tissue	with lymphoid tissue
	Day 1	
Hatching	91.70 ± 0.25	8.30 ± 0.25
	Day 10	
Experimental	84.89 ± 0.27	15.11 ± 0.27
Control	87.85 ± 0.14	12.15 ± 0.14
	Day 20	
Experimental	74.83 ± 0.34	25.17 ± 0.34
Control	77.79 ± 0.20	22.21 ± 0.20**
	Day 30	
Experimental	67.83 ± 0.11	32.17 ± 0.11
Control	70.73 ± 0.19**	29.27 ± 0.19
	Day 40	
Experimental	61.86 ± 0.12	38.14 ± 0.12
Control	66.29 ± 0.10	33.71 ± 0.10*
	Day 50	
Experimental	59.21 ± 0.28**	40.79 ± 0.28
Control	63.88 ± 0.12**	36.12 ± 0.12*

***Note**: *P < 0.05; **P < 0.01; ***P < 0.001 compared to the corresponding previous indicator*
***Source**: developed by the authors*

In the 10-day age period, in both groups, active development of pre-nodules was observed in diffuse lymphoid tissue, which were denser clusters of lymphocytes without a pronounced shell and clear contours (Fig. 4A), and in the poultry of the experimental group, the third level of structural organisation was also recorded – primary lymphoid nodules with dense cell cooperation. The reticular base of the latter had large-and small-sized architectonics in their central part due to the interweaving of reticular fibres, while collagen

fibres were not found there. At the periphery of the nodules, reticular fibres, together with collagen fibres, were part of the shell and were oriented in a circle.

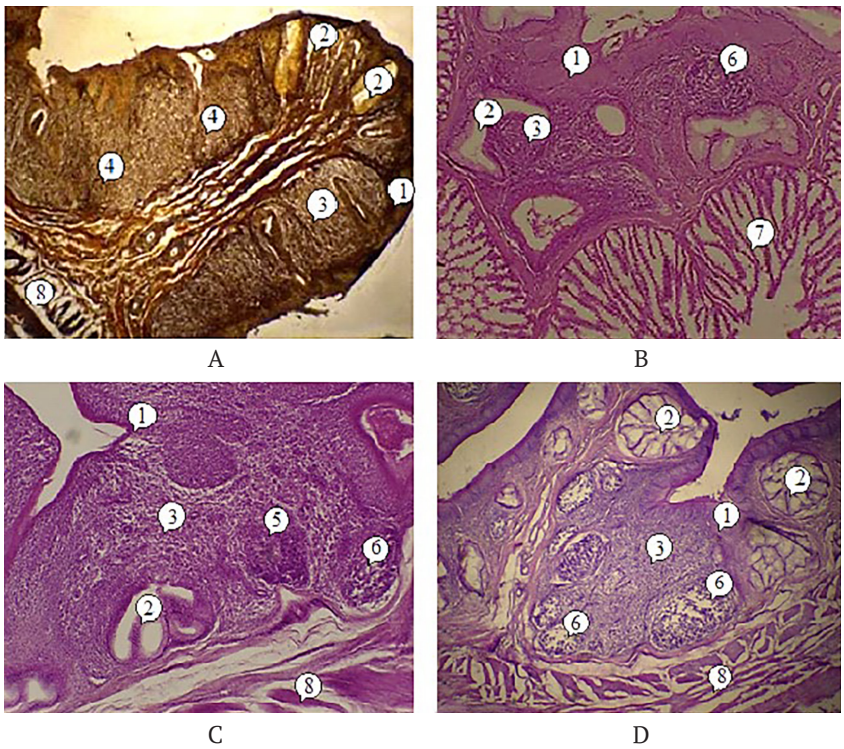


Figure 4. Area of transition of the oesophagus to the glandular part of the stomach of turkeys
***Note**: control group: A – 10-day and B – 20-day age; experimental group: C – 20-day and D – 30-day age. 1 – superficial epithelium; 2 – oesophageal glands; 3 – diffuse lymphoid tissue; 4 – pre-nodular form; 5 – primary lymphoid nodule; 6 – secondary lymphoid nodules; 7 – lobule of the deep gland of the glandular part of the stomach; 8 – muscle membrane. Impregnation with silver nitric acid according to Kelemen ×80 (A). Staining with haematoxylin and eosin ×80 (B, C, D)*
***Source**: developed by the authors*

In 20-day-old turkeys, the area of lymphoid tissue in the oesophageal tonsil mucosa increased by 66.58% (1.7 times) in the experimental group and by 82.80% (1.8 times, $P < 0.01$) in the control group compared to the corresponding indicators of the previous group (Table 2). Clusters of lymphoid tissue had different shapes and sizes and were separated from each other by structural elements of loose fibrous connective tissue. They included significant accumulations of diffuse lymphoid tissue, pre-nodules, and primary and secondary lymphoid nodules in birds of both groups (Fig. 4B, 4C). The latter are the fourth level of structural organisation with noticeably pronounced germinal (light) centres, which according to V.R. Indu *et al.* (2020), can increase in size with a significant antigenic effect on the body. Their appearance, according to the researchers, indicates a high immunological competence of the lymphoid tissue and, accordingly, the oesophageal tonsil. Individual secondary lymphoid

nodules were in the form of longitudinally arranged chains. Such chains lay in the thickness of the mucous membrane and were located both on the tops of the longitudinal folds and between them. Perivascular lymphoid nodules were registered in the submucosal membrane around the blood vessels. In numerous mitotic figures of lymphoid cells, mainly with light cytoplasm and macrophages, were observed in the breeding centres.

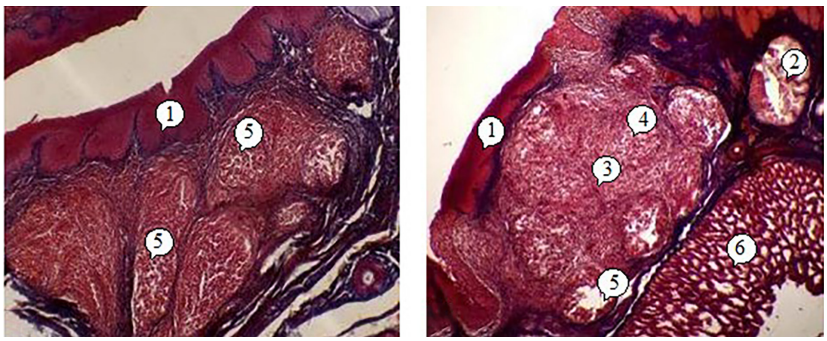
On the periphery of the secondary nodules, a mantle with densely arranged lymphocytes, mostly small in shape, was clearly visible, which were arranged in several layers and formed a thin strip around the breeding centre, which was also reported by C. Casteleyn *et al.* (2010) when studying the immune formations of the digestive tube of chickens. The researchers noted that small lymphocytes of the mantle zone have an immunological memory, that is, when they meet the antigen again, they react much faster and stronger, so they can fight bacteria and viruses. In the current study, in individual nodules, the area of the mantle that was turned to the epithelial layer of the mucous membrane was expanded and had the appearance of a dome. The latter was one of the sources of lymphocytes that migrated to the thickness of the surface epithelium. The formed secondary lymphoid nodules of the oesophageal tonsil of turkeys are limited to collagen, elastic and reticular fibres that formed their shell (capsule). Individual fibres had a connection with such fibres of their own plate of the mucous membrane. In the central part of the secondary nodules, the fibres were sparse and fragmented, and in most of them they were absent altogether. According to research data by C. Ceccopieri & J.P. Madej (2024), proliferation centres in lymphoid nodules occur only when the body's immunological activity increases in response to antigen penetration and the need for the production of lymphocyte populations increases.

On day 30, the area of lymphoid tissue in the mucous membrane continued to increase by 27.81% (1.2 times) in the experimental group and by 31.79% (1.3 times) in the control group compared to the previous age period (Table 2, Fig. 4D). The growth of infiltrated areas of the surface epithelium of the mucous membrane by lymphoid cells was observed. Individual lymphoid nodules were registered in the wall of the terminal parts of the oesophageal glands and their excretory ducts. Moreover, infiltration of glandular epithelial lymphocytes and their active migration as part of secretions to the surface of the oesophagus were observed. These data are consistent with the conclusions by I. Oláh *et al.* (2014) and A.M. Abdellatif1*et al.* (2022), that the replacement of the oesophageal glands with lymphoid tissue is not accidental, since this connection causes the production of antibodies in the form of immunoglobulin A, which together with the secretory component of the glands provide local resistance to infections on the surface of the mucous membranes.

In turkeys on days 40 and 50, the area of accumulations of lymphoid tissue in the mucous membrane increased by 18.55% (1.2 times) and 6.95% (1.0 times) in the experimental group and by 15.17% (1.1 times, $P < 0.05$) and 7.15% (1.0 times, $P < 0.05$) in the control group compared to the corresponding indicators of the previous period (Table 2). In these age groups, all levels of structural organisation of lymphoid tissue were observed, among them a significant number of secondary lymphoid nodules with well-defined light centres were noted (Fig. 5). The area of interepithelial infiltration by lymphocytes of epithelial tissue (superficial and glandular) also increased.

As noted above, with increasing age of turkeys, the area of lymphoid tissue in the mucous membrane of the oesophageal tonsil area increased. During the entire study period of poultry (from day-old to 50-day-old), its content

increased by 391.44% (4.9 times) in the experimental group and by 335.18% (4.3 times) in the control group, while the area of the mucous membrane without lymphoid tissue decreased (by 35.43%, 1.5 times in the experimental group and by 30.34%, 1.4 times in the control group) (Table 2). The most intensive increase in the area of lymphoid tissue was observed in birds of the experimental group from daily to 10-day age (by 82.05%, 1.8 times), and in the control group – from 10 to 20 days (by 82.80%, 1.8 times). With a lower intensity, this indicator increased in poultry of both groups from 40 to 50 days by 6.95% (1.0 times) in the experimental group and by 7.15% (1.0 times) in the control group.



A B

Figure 5. Area of transition of the oesophagus to the glandular part of the stomach in 50-day-old turkeys

***Notes**: A – control group; B – experimental group. 1 – superficial epithelium; 2 – oesophageal gland; 3 – diffuse lymphoid tissue;4 – primary lymphoid nodule; 5 – secondary lymphoid nodules; 6 – lobule of the deep gland of the glandular part of the stomach. Staining according to the Mallory method ×80 (A, B)*

***Source**: developed by the authors*

The content of individual structural and functional levels of oesophageal tonsil lymphoid tissue in turkeys of both studied groups changed with increasing age (Table 3). The largest area was the diffuse form of lymphoid tissue, which on the first day (after hatching) was 100%. With an increase in the age of turkeys (from day-old to 50-day age), the area of diffuse lymphoid tissue decreased by 47.83% (1.9 times) in the experimental group and by 41.83% (1.7 times) in the control group. The sharpest decrease in this indicator was observed in poultry from 10 to 20 days by 24.33% (1.3 times) in the experimental group and by 22.94% (1.2 times) in the control group.

Table 3. Content of structural levels of lymphoid tissue in the oesophageal tonsil in turkeys, %, M ± m, n = 6

Poultry group	Diffuse form	Pre-nodes	Lymphoid nodules	
			primary	secondary
		Day 1		
Hatching	100	-	-	-
		Day 10		
Experimental	88.50 ± 0.67	5.33 ± 0.50	6.17 ± 0.56	-
Control	94.82 ± 0.29	5.18 ± 0.29**	-	-

Table 3. Continued

Poultry group	Diffuse form	Pre-nodes	Lymphoid nodules	
			primary	secondary
		Day 20		
Experimental	66.97 ± 0.97	5.98 ± 0.33*	8.32 ± 0.50	18.73 ± 1.05
Control	73.07 ± 0.33	5.57 ± 0.15**	7.10 ± 0.34*	14.26 ± 0.23
		Day 30		
Experimental	62.38 ± 1.27	6.08 ± 0.42**	9.08 ± 0.21*	22.46 ± 0.88
Control	70.13 ± 0.44	6.03 ± 0.17***	7.76 ± 0.25**	16.08 ± 0.16*
		Day 40		
Experimental	56.35 ± 0.89	6.53 ± 0.28***	12.94 ± 0.59	24.18 ± 0.58**
Control	65.00 ± 1.72	6.20 ± 0.28**	9.76 ± 0.91***	19.04 ± 1.11*
		Day 50		
Experimental	52.17 ± 0.66	7.05 ± 0.52*	13.73 ± 0.28*	27.05 ± 0.59
Control	58.17 ± 0.50	6.87 ± 0.23**	11.92 ± 0.22	23.04 ± 0.75

***Note**: *P < 0.05; **P < 0.01; ***P < 0.001 compared to the corresponding previous indicator*
***Source**: developed by the authors*

Against the background of a decrease in the area of diffuse lymphoid tissue, an increase in the content of pre-nodules and lymphoid nodules was observed. As noted above, in turkeys of the experimental group, pre-nodules and primary lymphoid nodules were detected from 10-day age, and in the control group, pre-nodules were recorded from 10-day age, primary nodules – from 20-day age of the bird. In both groups, secondary lymphoid nodules were recorded from day 20. Moreover, in the poultry of the experimental group, the area of pre-nodules, primary and secondary lymphoid nodules was slightly larger than in the turkeys of the control group. The content of pre-nodules (from 10 to 50 days) increased by 1.3 times, or by 32.27% – in the experimental and 32.63% – in the control groups. This process was most intense in birds of the experimental group from 10 to 20 days by 12.20% (1.1 times, $P < 0.05$), and in turkeys of the control group – from 40 to 50 days by 10.81% (1.1 times, $P < 0.01$). The content of lymphoid nodules (primary and secondary) reached its maximum values in 50-day-old poultry. Notably, there are slightly more secondary lymphoid nodules in turkeys of both groups than in primary ones. A particularly significant increase in the area of primary lymphoid nodules was observed in poultry from 30 to 40 days (by 42.51% in the experimental and 25.77%, $P < 0.001$ in control groups), and with a lower intensity, this indicator in the experimental bird increased from 40 to 50 days by 6.11% ($P < 0.05$), and in the control – from 20 to 30 days at 9.29% ($P < 0.01$). The maximum increase in the content of secondary lymphoid nodules was recorded from 20 to 30 days in the experimental group (by 19.91%, 1.1 times) and from 40 to 50 days in poultry of the control group (by 21.01%, 1.2 times). With less intensity, this indicator increased from 30 to 40 days of age of experimental turkeys (by 7.66%, $P < 0.01$) and from 20 to 30 days (by 12.76%, $P < 0.05$) in control group.

The lymphoid nodules of the oesophageal tonsil of turkeys were mainly rounded and oval in shape, and some with were pear-shaped, egg-shaped, etc. (Fig. 6). They clearly showed the shell that separated the cellular composition of nodules from diffuse lymphoid tissue. Nodules

had different sizes and formed groups that are best expressed in birds of 40-50 days. Moreover, in both groups, the size of secondary nodules prevailed above the primary ones (Table 4).

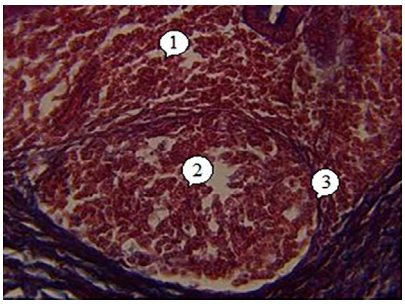


Figure 6. Lymphoid nodule of the oesophageal tonsil in the control group in 50-day-old turkeys
***Notes**: 1 – diffuse lymphoid tissue; 2 – secondary lymphoid nodule; 3 – collagen fibres. Staining according to the Mallory method ×100*
***Source**: developed by the authors*

Table 4. Size of lymphoid nodules of the oesophageal tonsil in turkeys, M ± m, μm (n = 6)

Poultry group	Lymphoid nodules					
	Primary			Secondary		
	Rounded	Oval		Rounded	Oval	
		Length	Width		Length	Width
			Day 10			
Experimental	118.83 ± 1.58	225.83 ± 3.86	149.83 ± 6.44	-	-	-
Control	-	-	-	-	-	-
			Day 20			
Experimental	169.50 ± 1.96	250.33 ± 4.85	163.50 ± 7.38	174.83 ± 1.99	262.33 ± 2.98	177.17 ± 5.29
Control	158.17 ± 3.30	221.50 ± 5.97	144.83 ± 2.55	164.17 ± 4.92	236.83 ± 3.14	147.67 ± 3.55
			Day 30			
Experimental	183.83 ± 4.02	263.00 ± 2.24	170.50 ± 1.21	187.33 ± 1.43	274.33 ± 4.79	179.00 ± 3.74*
Control	167.33 ± 4.42	237.83 ± 5.17	156.83 ± 4.02	179.5 ± 1.96	272.5 ± 3.27	160.17 ± 3.67
			Day 40			
Experimental	207.5 ± 7.56	274.17 ± 3.42	177.33 ± 5.67	221.83 ± 5.11	277.5 ± 4.76*	180.17 ± 4.20
Control	194.67 ± 2.80	269.5 ± 2.71	161.83 ± 4.57*	210.0 ± 2.24	274.0 ± 5.04*	171.67 ± 4.29
			Day 50			
Experimental	244.33 ± 9.71	286.17 ± 2.30	189.83 ± 5.14	250.17 ± 7.41	301.17 ± 4.76	191.17 ± 6.07
Control	201.83 ± 3.83*	277.67 ± 2.05	173.83 ± 10.55	233.17 ± 4.76	294.17 ± 2.65	178.67 ± 5.48

***Note**: *P < 0.05; **P < 0.01; ***P < 0.001 compared to the corresponding previous indicator*
***Source**: developed by the authors*

With an increase in the age of the bird, a gradual increase in the values of these indicators was observed. Over the period from 10 to 50 days, linear measurements (diameter of rounded, length and width of oval) of primary lymphoid nodules of experimental turkeys increased by 105.61% (2.1 times), 26.72% (1.3 times), and 26.69% (1.3 times), respectively, and the same indicators in poultry of the control group (20 to 50 days), respectively, by 27.60% (1.3 times), by 25.36% (1.2 times), and 20.02% (1.2 times). The diameter of rounded and

the length and width of oval primary lymphoid nodules in the experimental group increased with greater intensity from 10 to 20 days (corresponding to 42.64%, 10.85%, and 9.12%), while in the control group the width of oval nodules increased most intensively from 20 to 30 days (by 8.29%), and the diameter of rounded and oval length from 30 to 40 days (by 16.34% and 13.32%, respectively). Linear indicators of secondary lymphoid nodules of the oesophageal tonsil also increased with increasing age. Thus, during the period from 20 to 50 days of age, the diameter of rounded, length and width of oval nodules in the experimental group of poultry increased by 43.09% (1.4 times), 14.81% (1.1 times), and 7.90% (1.1 times), respectively, and the same indicators in turkeys of the control group, respectively, by 42.03% (1.4 times), 24.21% (1.2 times), and 20.99% (1.2 times). With a higher intensity of growth in the diameter of rounded secondary lymphoid nodules in poultry of the experimental group occurred from 30 to 40 days (by 18.42%), and the length and width of oval ones – from 40 to 50 days (by 8.53% and 6.11%, respectively), while in the control group the length and width of oval ones increased most intensively from 20 to 30 days (by 15.06% and 8.46%, respectively), and the diameter of rounded ones from 30 to 40 days (by 16.99%). Thus, the paper presented the features of the morphology of the oesophageal tonsil in vaccinated and unvaccinated turkeys, the stages of development of lymphoid tissue, and the established age period of the bird, in which its morphofunctional maturity occurs, which should be taken into consideration by specialists when drawing up treatment and preventive plans at poultry complexes.

Conclusions

The topography and structural and functional organisation of the lymphoid tissue of the oesophageal tonsil of vaccinated and unvaccinated Big-6 turkeys aged from one to 50 days old, and the timing of its morphofunctional maturity was determined, which specialists should consider when developing new vaccine prevention strategies. However, secondary lymphoid nodules were detected on day 20 in turkeys of both groups under study, which indicated that the oesophageal tonsil acquired morphofunctional maturity and the ability to exert a specific immune response to the action of foreign antigens. Secondary lymphoid nodules had well-defined light centres, in which lymphoid cells were recorded, including immunoblasts with light cytoplasm and macrophages. The lymphoid tissue of the oesophageal tonsil of birds of both groups was found in its own mucosal plate and submucosal base, while it was absent in the muscular and serous membranes. Some of the lymphocytes migrated to the lower layers of the surface epithelium and ended up around the blood vessels, in the wall of the secretory parts of the oesophageal glands between the glandulocytes and their excretory ducts. In this case, active migration of these cells as part of the secretory component to the surface of the mucous membrane was observed. With an increase in the age of turkeys in both groups, there was an uneven increase in the area of the mucous membrane with lymphoid tissue, which in 50-day vaccinated poultry was 11.45% (1.1 times) more than in unvaccinated poultry. The content of diffuse form in the lymphoid tissue decreased, and pre-nodules and lymphoid nodules (primary and secondary) increased. With increasing age of turkeys, the size of lymphoid nodules increased, which acquired maximum values in 50-day-old poultry and were 17.39% (rounded primary) and 6.79% (rounded secondary) in the vaccinated group were larger than in the unvaccinated one. In addition, in turkeys of both groups, the linear indicators of secondary nodules exceeded those of primary nodules.

Thus, it was found that vaccination of poultry against bacterial and Infectious diseases stimulates the development of structural levels of the lymphoid tissue of the oesophageal tonsil, which accelerates its morphofunctional maturity. Morphological and morphometric data also indicate a close relationship between age-related changes and vaccination with the formation of immunocompetent structures of the oesophageal tonsil of turkeys and confirm its important role in ensuring local immunity. Prospects for further research can be aimed at investigating the structural and functional organisation of the oesophageal tonsil of older turkeys, the impact of vaccination on it, and comparative assessment with other representatives of the animal world.

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Conflict of Interest

None.

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Макро- та мікроструктура стравохідного мигдалика індиків у поствакцинальний період

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Анотація. Успішний розвиток птахівництва залежить від стану імунної системи птиці, яка з перших діб життя контактує зі значною кількістю антигенів, що потрапляють в організм переважно через травний канал і провокують розвиток захворювань. Затримка корму в перехідній зоні між стравоходом і шлунком призводить до формування стравохідного мигдалика, як одного з найбільш розвинутих імунних утворень. Метою цієї роботи було з'ясувати розвиток стравохідного мигдалика індиків і встановити строки його повної морфофункціональної зрілості за вакцинопрофілактики. Матеріал для проведення макро- та мікроскопічних досліджень відібрали від 66 особин індиків породи Біг-6 на ранніх етапах постнатального періоду онтогенезу, яких розділили на дослідну і контрольну групи. При виконанні роботи використовували класичні методи морфологічних досліджень. Показано, що в обох групах індиків рівні структурної організації лімфоїдної тканини, яка формує основу стравохідного мигдалика, виникають у певній послідовності, але з різною інтенсивністю. У добової птиці виявляється перший її рівень – дифузна лімфоїдна тканина, яка представлена локальними скупченнями дифузно розташованих лімфоцитів, частина яких мігрує у поверхневий епітелій і має тісний контакт з епітеліоцитами. На 10 добу індиків дослідної групи з'являлися передвузлики із щільним розташуванням лімфоцитів без оболонки (другий рівень) і первинні лімфоїдні вузлики з компактним розташуванням клітин лімфоїдного ряду, що обмежені оболонкою (третій рівень), а з 20 доби – вторинні лімфоїдні вузлики зі світлими центрами і добре вираженою мантійною зоною (четвертий рівень), тоді як у контрольної групи – передвузлики реєструвалися з 10-добового, а первинні і вторинні лімфоїдні вузлики – з 20-добового віку. Це може свідчити про те, що на 20 добу стравохідний мигдалик як імунне утворення набуває морфофункціональної зрілості, а його клітини здатні розпізнавати і знищувати конкретні антигени. Отримані результати сприяють з'ясуванню природних механізмів розвитку імунологічних процесів у птиці в онтогенезі, що слід враховувати ветеринарним лікарям при розробці нових стратегій вакцинопрофілактики

Ключові слова: ділянка переходу стравоходу у шлунок; лімфоїдна тканина; лімфоїдні вузлики; морфологічні дослідження; лімфоцити; птиця

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Investigation of the ability to form biofilms *in-vitro* in sanitary-indicatory bacteria isolated from chickens

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Abstract. Biofilms provide resistance to antimicrobial agents and the body's immune response in microorganisms that colonise the digestive tract of animals, in particular poultry. The study of biofilm formation of indicatory bacteria isolated from chickens under different keeping conditions allows assessing the impact of environmental factors on their phenotypic adaptation and potential risk to animal and human health. The purpose of the study was to determine the ability of *Escherichia coli* and *Enterococcus faecalis* bacteria isolated from chickens kept in a vivarium and on free range to form biofilms. Bacteriological, morphological, biochemical, and microscopic research methods were applied. The intensity of biofilm formation in indicatory microorganisms was assessed by the adsorption/resorption index of a 0.1% solution of crystal violet using polystyrene Petri dishes. The optical density was measured spectrophotometrically at a wavelength of 570 nm. It was found that *E. coli*, *E. faecalis*, *Klebsiella spp*. and *Pseudomonas aeruginosa* were isolated in samples from chickens kept in a vivarium, while in free-range chickens, representatives of the genus Klebsiella and Pseudomonas aeruginosa were not detected, indicating a lower presence of potential pathogens in natural conditions. All the cultures under study formed low- or medium-density biofilms. For *E. coli* isolates obtained from free-range chickens, the average value $\lambda = 0.264 \pm 0.09$, while for vivarium isolates – $\lambda = 0.187 \pm 0.07$. Cultures of *E. faecalis* biofilms were formed with an intensity of $\lambda = 0.217 \pm 0.04$ in free-range chickens and $\lambda = 0.137 \pm 0.03$ in vivarium chickens. Consequently, isolates obtained from natural conditions were characterised by a higher intensity of biofilm formation – by 41.2% (*E. coli*) and 58.4% (*E. faecalis*) in comparison with the conditions of a controlled microclimate. This may indicate a stimulating effect of environmental factors on the expression of adhesion and biofilm formation genes. However, all cultures under study were isolated from clinically healthy chickens, which indicates a commensal nature of the microbiome. The results obtained are important for assessing the risks of horizontal transfer of resistance genes and the formation of stable microbial biofilms in poultry farming

Keywords: indicatory bacteria; *Escherichia coli*; *Enterococcus faecalis*; poultry keeping conditions; phenotypic adaptation; sanitary and microbiological monitoring

Introduction

With the development of poultry farming in the world, subclinical forms of infections pose a significant threat to poultry health and the stability of production processes. The high concentration of livestock, intensive rearing technologies, and constant pressure from bacterial and viral pathogens require strengthening comprehensive veterinary control measures. In Ukraine, poultry farming remains the leading branch of animal husbandry, but its further development is primarily determined by epizootic well-being, the level of biosafety, and the quality of preventive programmes. Key factors in the industry's sustainability include effective monitoring of infectious agents, compliance with veterinary and sanitary requirements, optimisation of vaccination strategies, and timely detection of pathogens. It is veterinary support and control of zoonotic risks that determine the ability of poultry farms to resist infectious threats and ensure stable production.

J. Li *et al.* (2024) proved that an important component of maintaining the health and productivity of poultry is the state of the intestinal microbiome. It plays a key role in regulating digestion, converting nutrients, developing and maturing the immune system, maintaining intestinal barrier functions, and developing

resistance to pathogens. The gut microbiota is in dynamic balance with the host body, forming a complex system of interactions that determines the overall physiological stability of the body. It was established that the keeping conditions – in particular, stocking density, microclimate, stress factors, diet, and microbial pollution of the environment can significantly change the species composition of the microbiota and affect its functional activity.

L. Vygovska *et al.* (2025) found out that the international organisation FAO pointed out the existence of an intensive, subintensive, and extensive poultry rearing system, and household maintenance actually formed a separate group with the lowest level of biosafety. This is conditioned by the fact that owners of small farms, as a rule, are not aware of measures aimed at preventing the introduction and spread of infections in the herd. Under such conditions, the risk of circulation of bacterial pathogens that are dangerous to both poultry and humans increases. The most common among them are *Escherichia*, enterococci, and other conditionally pathogenic microorganisms. According to S.C. Pinto *et al.* (2022) and O. Bezpalko *et al.* (2024), the absence of systematic veterinary prevention contributes not only to the disease of poultry, but also creates conditions for the transmission of zoonotic infections to humans, especially in direct contact of owners with livestock. Thus, domestic poultry farming is a potential reservoir of pathogenic microorganisms and may be a risk factor for public health, which requires increased attention within the framework of the Ukrainian state strategy for ensuring biological safety and biological protection "One Health" (Order of the Cabinet of Ministers of Ukraine No. 1416-r, 2019).

In the context of contemporary approaches to biosafety and the concept of "One Health", the study of biofilm formation in sanitary-indicatory bacteria that are constantly present in the microbiota of farm animals is of particular importance. It is such microorganisms that, under certain conditions, can become a reservoir of antibiotic resistance genes and a factor of horizontal transfer of pathogenicity determinants. The conditions of keeping poultry affect the level of phenotypic adaptation of these bacteria, including the ability to form biofilms. S. Sharma *et al.* (2023) proved that bacteria in biofilms have distinctive features from microbes in the planktonic state, showing increased antimicrobial resistance, avoidance of host immune factors, and increased resistance to adverse environmental factors. The presence of such signs in pathogenic bacteria contributes to a more severe course of the disease and longer treatment. As confirmed by M. Cordero *et al.* (2023), flagella, pili, and fimbriae are responsible for the beginning of the bacterial biofilm formation process, which provided primary contact between the microorganism and the epithelial cells of the macroorganism.

M. del Mar Cendra & E. Torrents (2021) noted that microorganisms aggregated on the surface synthesise extracellular polymer substances, extracellular matrix, extracellular DNA, and binding proteins to surrounding bacteria, including a number of regulatory factors (system of QS – quorum sensors), which affect the formation and destruction of biofilms. K. Xiaoxia *et al.* (2023) described the mechanisms of biofilm formation, their role in chronic, nosocomial, and medical infections, and the increased resistance of bacteria in biofilms to antibiotics. It was indicated that because of this resistance, conventional antibiotic therapy is often ineffective. The study highlighted that elimination of biofilm infections requires innovative technologies, not just classical antibiotics.

Thus, the analysed studies on the problems biofilm formation in microorganisms indicate that the ability of bacteria to form biofilms is one of the key mechanisms of their survival, colonisation, and pathogenicity. Biofilms provide microorganisms with protection against host immune

system factors and antimicrobial actions, contribute to the development of chronic infections, and can play a crucial role in the conservation and transmission of pathogens in poultry populations. Simultaneously, the level of biofilm formation is a variable characteristic that depends on the type of microorganism, environmental conditions, and factors of keeping poultry. This highlights the need to study the phenotypic features of biofilm formation in sanitary-indicatory bacteria isolated from poultry under different containment systems, since such microorganisms can act as a reservoir of resistance genes and potential determinants of pathogenicity. The data obtained are important for assessing epizootological risks in poultry farming and improving biosafety measures. Therefore, the purpose of this study was to compare the ability to form biofilms with sanitary-indicatory microorganisms isolated from chickens under different keeping conditions. The objectives of the study were: to isolate and identify sanitary-indicatory bacteria from cloacal flushes of chickens raised in various conditions; to assess the ability of isolates to form biofilms *in vitro*; to conduct a comparative analysis of biofilm formation between isolates and determine its possible relationship with the keeping conditions of chickens.

Materials and Methods

Microbiological studies were conducted throughout 2024 in the scientific laboratory of the Department of Veterinary Epidemiology and Animal Health, Faculty of Veterinary Medicine, National University of Life and Environmental Sciences of Ukraine, Kyiv, Ukraine. Biomaterial for research (faecal samples) was collected from clinically healthy chickens, 77 animals of 30-35-day-old Cobb 500 crossbreeds. In the vivarium of the Faculty of Veterinary Medicine of the National University of Life and Environmental Sciences of Ukraine (Group 1), chickens (n = 30) were kept in a standard KR 108 collapsible cage designed for laying hens and broilers. Chickens received a standard balanced diet and local optimal climatic conditions (temperature 30 ± 3°C, humidity 55 ± 5%). On the household plot (Group 2) located within one of the amalgamated territorial communities of the Kyiv Oblast, chickens (n = 47) were kept free-range, fed a prepared feed mixture of crushed and steamed wheat and corn, kitchen waste, and given unlimited access to water.

Cloacal flush samples (10 samples per group) from clinically healthy chickens were delivered in a thermal container at 2-8°C in accordance with DSTU 8703-1:2017 (2017) and DSTU 8703-2:2017 (2017). Bacteriological studies to determine the morphological and biochemical characteristics of the isolated isolates and to establish the intensity of biofilm formation were conducted in the Research Laboratory of Epizootology and Infectious Diseases of the Faculty of Veterinary Medicine using certified culture media and equipment in accordance with current standards (Table 1).

Table 1. Methods and standards for sample preparation, isolation and identification of microorganisms

Research stage	Microorganisms / indicators	Method / regulatory document
Preparation of samples and dilutions	Test samples, initial suspension, tenfold dilutions	ISO 6887-1:2017 (2017)
Selection and identification	*Salmonella spp.*	ISO 6579-1:2017 (2017)
Selection and identification	*Pseudomonas aeruginosa*	Classical bacteriological method
Selection and identification	*Listeria spp.*, *Listeria monocytogenes*	ISO 11290-1:2017 (2017)
Selection and identification	*Yersinia enterocolitica*	ISO 10273:2017 (2017)

Table 1. Continued

Research stage	Microorganisms / indicators	Method / regulatory document
Determination of MPN	Enterobacteria, *Escherichia coli*, *Klebsiella spp.*	ISO 21528-1:2017 (2017)
Determination of MPN	Enterococci	DSTU 8534:2015 (2015)

***Note**: MPN – most probable number. The method for determining the most probable number provided for the use of the MPN table with a 95% confidence interval and an appropriate formula for calculating the number of microorganisms*
***Source**: compiled by the authors*

To clarify morphological features, a generally accepted bacteriological approach was used, namely: cultivation on liquid and solid nutrient media. Bacteriological examination was performed by seeding cloacal flushes with a sterile swab on a nutritious broth. Cultivation was carried out at the optimal temperature for growth – 37°C, for 24 hours. Isolation and identification of a wide range of bacterial pathogens was performed for a comprehensive sanitary and microbiological assessment of cloacal flushes and confirmation of the absence of clinically significant pathogens. 9 isolates of *E. coli* and *E. faecalis* were selected for further study of the biofilm formation intensity as typical representatives of the intestinal microbiota of poultry and model objects of biofilm studies.

The cultures obtained on nutrient broth were transferred with a bacteriological loop using frequent broad strokes into separate Petri dishes with Endo agar and xylose-lysine-deoxycholate agar (XLD), covering the entire surface of the agar, and then incubated in a thermostat for 24 hours at 37°C. Subsequently, to obtain a pure culture, individual representative colonies were extracted from the agar surface using a bacteriological loop, transplanted into a nutrient broth with meat and peptones and slant nutrient agar with meat and peptones, and incubated at 37°C for 24 hours. Morphological properties were determined by microscopy of gram-stained smears, and typical growth was determined by inoculation of cultures on liquid and solid nutrient media.

To assess bacterial motility, isolated cultures were grown at 37°C in semi-liquid MPA (0.25-0.3%). Inoculation was performed by placing the sample in a column with semi-liquid agar. Mobility was also evaluated by microscopy of daily agar cultures using the "crushed drop" technique. The biochemical characteristics of the isolate were studied by inoculation with Hiss medium supplemented with various sugars (maltose, glucose, mannitol, sucrose, lactose, rhamnose, and raffinose). In addition, the ability to produce enzymes (ornithine decarboxylase, phenylalanine deaminase, lysine decarboxylase, arginine dehydrolase), urea, and indole was evaluated, and the Foges-Proskauer reaction was performed.

Indirect estimation of bacterial biofilm biomass by crystal violet adsorption/resorption was used, following the method described by S. Stepanovic *et al.* (2000) and M. Kukhtyn & N. Krushelnytska (2014). Biofilms were stained with a 0.1% aqueous solution of crystalline violet at 30°C for 60 minutes. Sterile MPB was used as a control. To obtain reliable data, the experiments were repeated four times. The optical density was determined spectrophotometrically at a wavelength of 570 nm. With an optical density value of less than 0.1, it was assumed that the cultures under study did not form a biofilm; from 0.1 to 0.49, the ability to form a biofilm was considered low; an optical density value between 0.5 and 1.0 indicated the formation of a biofilm of medium density; an optical density value of 1.0 and above indicated the formation of a high-density biofilm.

The results of the biofilm formation study were analysed using the SPSS Statistics software suite. Before making comparisons, the normality of the distribution of quantitative indicators was checked using the Shapiro-Wilk test. To compare indicators between groups, the following methods were used: in the case of a normal distribution, a parametric t-test for independent samples; in case of deviation from normality – a nonparametric Mann-Whitney test. The results were presented as mean ± standard deviation (SD). Differences at $P < 0.05$ were considered statistically significant. During the research, the recommendations of ARRIVE (n.d.) and Directive 2010/63/EU (2010) on the ethical treatment of animals used for scientific research were followed. The research was approved by the Bioethics Expertise Commission of the National University of Life and Environmental Sciences of Ukraine of Ukraine on 26 November 2024, No. 022.2024.

Results and Discussion

To assess the microbiological status of chickens kept in various conditions, bacteriological studies of cloacal flushes were conducted to identify zoonotically significant enterobacteria (*Salmonella spp., Listeria spp., Yersinia spp.*), which have potential epidemiological and veterinary sanitary significance. Based on the results of sowing followed by morphological, cultural, and biochemical identification, the pathogens of the listed genera were not detected in any of the samples studied in both experimental groups. Absence of *Salmonella spp., Listeria spp*. and *Yersinia spp.* indicates a satisfactory sanitary and epizootic condition in the premises where chickens were kept, a low probability of participation of chickens as reservoirs of zoonotic pathogens in these conditions, and the effectiveness of hygienic and preventive measures. This result is important from a practical standpoint, since the presence of *Salmonella spp.* or *Listeria spp.* in the number of young chickens is one of the key risk factors for food toxicoinfections and contamination of poultry products. In natural and small farms, the risk of introducing these pathogens remains traditionally higher due to the greater openness of the environment, but the data obtained do not confirm such a threat to the studied conditions.

Thus, the basic microbiological status of both groups of chickens can be regarded as successful, which allowed further focusing on analysing the composition of commensal microflora and studying its functional properties, in particular, the ability to form biofilms. Such results are consistent with data from EFSA and ECDC (2024), according to which the risks of contamination with *Salmonella spp.* and other pathogenic enterobacteria in young poultry significantly depend on the sanitary quality of feed and water, and the control of sources of infection in the first weeks of life. In the present study, chickens of both groups were in proper sanitary and hygienic condition, which may explain the absence of pathogens.

However, differences in the composition of the commensal microbiota were established. In samples from chickens from household farms (Group 2), 10 cultures of *Enterococcus spp.* and 10 cultures of *E. coli* were isolated and identified, which corresponded to the typical spectrum of normal intestinal microflora of poultry. In samples from chickens kept in vivarium conditions (Group 1), the spectrum of isolated microorganisms was wider: in addition to 10 cultures of *E. coli* and 10 cultures of *Enterococcus spp.*, 2 cultures of representatives of the genus *Klebsiella spp.* and 1 culture of *Pseudomonas aeruginosa* were also highlighted. The presence of these species was more often associated with conditions with increased humidity levels and longer microbial persistence on the surfaces of equipment and maintenance materials. This may reflect a more stable microecological

environment of the vivarium, where external factors vary less than in vivo.

The isolation of *Klebsiella spp*. and *P. aeruginosa* in Group 1 is important in terms of the potential opportunistic pathogenicity of these bacteria. According to R.P. Sequeira *et al*. (2020) and N.B.V. Tran *et al*. (2023), *Klebsiella spp*. and *P. aeruginosa* can remain part of the commensal microflora for a long time, but under conditions of immune stress or dysbiosis, they can exhibit pathogenic properties, in particular in birds – septicemia, respiratory lesions, and omphalitis. On the other hand, in free-range chickens, microbial diversity was less broad but ecologically stable, which was consistent with data on the priority of dominance of basic symbiotic taxa under more natural environmental conditions. Free range provided a fairly diverse microbial contact with the environment (soil, vegetation), but without pronounced conditions for the persistence of opportunistic bacteria, which often accumulate in closed technological systems.

Thus, the results obtained suggest that the keeping conditions of poultry can affect the structure of the commensal microbiome, but do not necessarily determine the presence of pathogenic microflora. In vivarium conditions, more diverse microbial associations with the inclusion of opportunistic bacteria can be formed, whereas in free-range conditions, the microbial profile was more stable and physiologically typical, represented by *E. coli* and *Enterococcus spp*. The differences in the microflora of cloacal swabs between groups of chickens were that when kept "free-range", no *Klebsiella spp*. and *P. aeruginosa* bacteria, which are potentially pathogenic to birds and humans, were detected, whereas in vivarium conditions they were isolated. This may indicate the influence of microbiological environment conditions on the development of the composition of commensal and opportunistic microbiota, and the possible role of content density and microclimate in the selection of microorganisms.

Subsequently, the ability of indicator commensal bacteria was evaluated – *E. coli* and *E. faecalis* isolated from chickens under different keeping conditions, to form biofilms, which were considered as one of the key factors of potential pathogenicity, colonisation ability, and resistance of bacterial populations. Thus, determination of the intensity of biofilm formation allowed assessing not only the ecological adaptive properties of microbes, but also the risks of their possible transformation into pathogenic forms due to stress factors or microbiome imbalance. To determine the intensity of biofilm formation, five *E. coli* isolates obtained from chickens in Group 2 and four *E. coli* isolates from chickens in Group 1 were selected and purified to pure cultures. Similarly, *E. faecalis* biofilm formation was later evaluated, which allowed for a parallel comparative characterisation between species. Each experiment was performed in 4 repetitions (n = 4), which ensured statistical reliability and reproducibility of the results. Results of estimation of optical density of *E. coli* biofilms at $\lambda = 570$ nm, are shown in Table 2.

Culture *E. coli,* isolated from Group 2 chickens, they showed wide variability in the intensity of biofilm formation – from 0.061 to 0.704, which indicates the existence of populations with different adaptive strategies. Some isolates were characterised by a weak level of biofilm formation, while others formed moderately dense biofilm matrices. The average value for the group (n = 5) was 0.264, which corresponds to a low or moderate level of biofilm formation.

Instead, *E. coli* isolates from Group 1 chickens had a smaller range of optical density fluctuations – 0.095-0.314, which may be due to the stability of microbiological and environmental influences, the absence of a wide range of external stressors, and limited contacts with natural bacterial associations. The average group value

was 0.187, which indicates mostly weak biofilm structures. The data obtained are consistent with the results of O. Bezpalko *et al.* (2024), where it was noted that free access to the natural microbial environment contributes to phenotypic variability and increases the ecological plasticity of commensal microorganisms. While *E. coli* isolates obtained under controlled retention conditions were more often characterised by standardised functional activity profiles.

Table 2. Indicators of optical density of biofilms formed by *E. coli*

Culture	Variant	*E. coli*, free-range chickens					*E. coli*, chickens kept in a vivarium			
		1	2	3	4	5	1	2	3	4
Cultivation medium, T°C		TSB, 37 ± 1°C								
	control	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003
	1	0.089	0.131	0.574	0.269	0.276	0.175	0.098	0.149	0.133
	2	0.074	0.218	0.492	0.147	0.160	0.205	0.126	0.202	0.118
	3	0.064	0.156	0.707	0.399	0.283	0.241	0.204	0.272	0.185
	4	0.129	0.230	0.655	0.159	0.117	0.209	0.172	0.317	0.237
λ 570, result with control	Range of values λ, Min-Max	0.064-0.129	0.131-0.230	0.492-0.707	0.147-0.399	0.117-0.283	0.175-0.241	0.098-0.204	0.149-0.317	0.118-0.237
	Average value, n = 4	0.089	0.184	0.607	0.244	0.209	0.208	0.150	0.235	0.168
	Range of average values λ, Min-Max	0.089-0.607					0.150-0.235			
	M (mean)	0.267 (n = 5)					0.1903 (n = 4)			
	M +/- m	0.27 ± 0.09					0.19 ± 0.02			
	1	0.086	0.128	0.571	0.266	0.273	0.172	0.095	0.146	0.130
	2	0.071	0.215	0.489	0.144	0.157	0.202	0.123	0.199	0.115
	3	0.061	0.153	0.704	0.396	0.280	0.238	0.201	0.269	0.182
	4	0.126	0.227	0.652	0.156	0.114	0.206	0.169	0.314	0.234
Actual value (λ 570 – control)	Range of values λ, Min-Max	0.061-0.126	0.128-0.227	0.489-0.704	0.144-0.396	0.114-0.280	0.172-0.238	0.095-0.201	0.146-0.314	0.115-0.234
	Average value, n = 4	0.086	0.181	0.604	0.241	0.206	0.205	0.147	0.232	0.165
	Range of average values λ, Min-Max	0.086-0.604					0.147-0.232			
	Average value of λ in the group	0.264 (n = 5)					0.187 (n = 4)			
	M +/- m	0.26 ± 0.09					0.19 ± 0.02			

***Note**: interpretation of optical density (OD) values: TSB – trypton-soy broth; OD < 0.1 – the culture does not form biofilms; OD from 0.1 to 0.49 – the culture forms low-density biofilms; OD from 0.5 to 1.0 – the culture forms medium-density biofilms; OD > 1.0 – the culture forms high-density biofilms*
***Source**: developed by the authors based on own research*

It is important to note that even moderate levels of biofilm formation in commensal isolates are important for the stability and balance of intestinal microbial communities, since biofilms act as a kind of ecological reservoir that ensures the colonisation stability of the population and its protection from competitive microbes and external stress influences. Thus, it was found that the keeping conditions of chickens play a significant role in the phenotypic manifestation of the ability of *E. coli* to the formation of biofilms. The revealed variability in the intensity of biofilm formation may indicate that this indicator is not a fixed species characteristic, but is formed as a response to the influence of external environmental factors. Free-range

conditions involving a wider range of microecological stimuli and microbial associations probably contributed to the activation of adaptive mechanisms associated with increased colonisation resistance and the formation of protective biofilm structures. In contrast, in the controlled microbiological environment of vivarium, where the influence of external factors and microbial competition was minimised, a decrease in the intensity of biofilm formation was observed, which can be considered as a result of a reduced need for structured forms of population survival.

The data obtained are consistent with current ideas about biofilm as a phenotypic manifestation of bacterial ecological adaptation, which is formed in response to changes in environmental parameters, such as the presence of competitive microorganisms, fluctuations in pH and temperature, and the availability of nutrients and substrates for adhesion (Gupta *et al*., 2016; Sonderholm *et al*., 2017). Thus, *E. coli* as part of the chicken microbiome, it not only acts as a commensal resident, but also demonstrates flexibility in choosing a survival strategy that is of important environmental and veterinary sanitary importance.

Analysis of the intensity of biofilm formation showed that among the *E. coli* isolates selected from Group 2 chickens, three cultures out of five formed low-density biofilms, one culture (*E. coli* No. 3) was characterised by the formation of medium-density biofilms, and the culture *E. coli* No. 1 did not form biofilms in three repetitions, whereas in one repetition it showed the formation of low-density biofilms (Table 2). In contrast, all *E. coli* isolates obtained from Group 1 chickens stably formed low-density biofilms in all study variants (n = 4), which indicated more uniform adaptive properties of the bacterial population under conditions of limited contact with natural microbiocenoses.

Further studies were aimed at evaluating the biofilm-forming ability of *E. faecalis* isolates and comparison of the results obtained with the data on *E. coli*, which allowed tracing interspecific differences in adaptive mechanisms associated with the development of biofilm structures in the composition of the resident gut microbiota of chickens. When evaluating the ability to form biofilms with isolates of *Enterococcus faecalis*, the study included 6 cultures isolated from Group 2 chickens and 2 cultures obtained from Group 1 chickens. As in the case of *E. coli*, each measurement was performed in 4 repetitions (n = 4), which ensured the reliability of the obtained comparative indicators. The results of determining the density of biofilms in these cultures are shown in Table 3.

Table 3. Indicators of optical density of biofilms formed by *E. faecalis*

Culture	Variant	*E. faecalis*, free-range chickens						*E. faecalis*, chickens kept in a vivarium	
		1	2	4	5	7	8	1	2
Cultivation medium, T°C		TSB, 37 ± 1°C							
λ 570	control	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003
	1	0.268	0.213	0.137	0.194	0.208	0.203	0.155	0.139
	2	0.209	0.246	0.151	0.217	0.201	0.209	0.166	0.103
	3	0.269	0.227	0.235	0.297	0.210	0.208	0.153	0.137
	4	0.207	0.242	0.254	0.215	0.250	0.204	0.168	0.105
λ 570, result with control	Range of values λ, Min-Max	0.207-0.269	0.213-0.246	0.137-0.254	0.194-0.297	0.201-0.250	0.203-0.209	0.152-0.168	0.103-0.139
	Average value, n = 4	0.238	0.232	0.194	0.230	0.217	0.206	0.160	0.121
	Range of average values λ, Min-Max	0.194-0.238						0.121-0.160	
	M (mean)	0.165						0.141	
	M +/- m	0.22 ± 0.01						0.14 ± 0.02	

Table 3. Continued

Culture	Variant	*E. faecalis*, free-range chickens						*E. faecalis*, chickens kept in a vivarium	
		1	2	4	5	7	8	1	2
Actual value of λ 570 (λ 570 - control)	1	0.265	0.210	0.134	0.191	0.205	0.200	0.152	0.136
	2	0.206	0.243	0.148	0.214	0.199	0.206	0.163	0.100
	3	0.267	0.224	0.232	0.294	0.207	0.205	0.156	0.132
	4	0.204	0.239	0.251	0.212	0.247	0.201	0.159	0.104
	Average value, n = 4	0.235	0.229	0.191	0.227	0.215	0.203	0.157	0.118
	Average value by keeping conditions	0.162						0.137	
	Range of average values λ, Min-Max	0.191-0.235						0.118-0.157	
	M (mean)	0.217 (n = 6)						0.137 (n = 2)	
	M +/- m	0.22 ± 0.01						0.14 ± 0.02	

***Note**: interpretation of optical density values (OD): TSB – trypton-soy broth; OSH < 0.1 – the culture does not form biofilms; OD from 0.1 to 0.49 – the culture forms low-density biofilms; OD from 0.5 to 1.0 – the culture forms medium-density biofilms; OD > 1.0 – the culture forms high-density biofilms*
***Source**: developed by the authors based on own research*

These results are consistent with data from studies by F. Lebreton *et al.* (2014) and C. Geraldes *et al.* (2022), who indicated that *E. faecalis*, despite their commensal status, can switch to an opportunistic phenotype under conditions of increased environmental pressure and inter-microbial competition. In particular, it was found that in natural and farm ecosystems *E. faecalis* more actively expresses surface attachment proteins (MSCRAMMs), genes of intercellular signalling systems, and factors involved in the synthesis of the polysaccharide matrix of biofilms. On the contrary, in a vivarium environment where a controlled microclimate, standardised feeding, and the influence of foreign microbial factors are minimised, the need for bacteria to form complex survival structures decreases. Therefore, isolates obtained from chickens kept in a vivarium were more likely to show low-intensity biofilm formation, reflecting the state of microbiological stability of the environment in which the host macroorganism is located. Thus, the identified differences characterise the biofilm not as a static feature of the species, but as a dynamic adaptation phenotype formed in response to environmental challenges. This confirms the concept of ecological plasticity of the microbiome and emphasises that the keeping conditions are an important factor that can modulate the pathobiological properties of commensal bacteria.

Analysis of the obtained indicators of optical density of biofilms shows that all eight studied cultures of *E. faecalis* formed low-density biofilms. This uniformity of biofilm formation intensity indicates a relatively uniform potential of commensal enterococci for adhesion and formation of microbial consortia on surfaces. This is consistent with their physiological role as permanent components of the avian gut microbiota.

In previous studies, O. Bezpalko *et al.* (2024) found differences in the composition of indicatory bacteria in chicken droppings depending on the conditions of poultry rearing. Thus, zoonotic pathogenic bacteria were not detected in free-range chickens, while such bacteria were present in poultry raised in simulated conditions of an industrial poultry house with controlled microclimate parameters and a standard diet. This determined the need for further study not only of the species structure of the microbiota, but also of the biological properties

of commensal bacteria, in particular, the ability to form biofilms as an important factor in their resistance and potential pathogenicity. The current study focused on comparing properties of *E. coli* and *E. faecalis* isolated from chickens raised in different keeping conditions.

Biofilm formation, according to the data by S. Sharma *et al.* (2023), is a key mechanism for bacterial survival and the development of their antibiotic resistance. That is why the study of the intensity and features of biofilm formation by indicatory bacteria in the poultry intestine is important for understanding the mechanisms of their potential pathogenicity and role in the development of dysbiosis. *E. coli* cultures isolated from Group 2 chickens formed low-density biofilms with an average optical density value $\lambda = 0.264$. Similarly, *E. coli* isolates obtained from Group 1 chickens also formed low-density biofilms, but the average value for this group was lower and was 0.187 (which is 29.2% less than the average optical density of the *E. coli* isolate from free-range chickens kept on a private plot). Culture of *E. faecalis* isolated from free-range chickens formed low-density biofilms with an average value of $\lambda = 0.217$, while enterococci isolated from chickens kept in a vivarium were characterised by a lower average biofilm density – 0.137.

A comparative analysis of the results obtained shows that the ability of microorganisms to form biofilms can vary depending on the external conditions of the host macroorganism. In particular, *E. coli* and *E. faecalis* isolates from free-range chickens were characterised by higher values of optical density of biofilms compared to isolates from poultry in standardised vivarium conditions. Thus, the average level of biofilm formation in the group *E. coli* from Group 2 was 41.17% higher, and *E. faecalis* – 58.4% higher compared to the corresponding Group 1 chicken cultures.

These data are consistent with the idea of biofilm as a form of phenotypic adaptation of bacteria to environmental factors. M. Sonderholm *et al.* (2017) emphasised that biofilm organisation is an evolutionarily formed mechanism of resistance to changes in the physical and chemical parameters of the environment. Similar conclusions were given by C. de la Fuente-Núñez *et al.* (2013), who considered biofilm formation as a form of collective bacterial behaviour activated by external stress. P. Gupta *et al.* (2016) proved the dependence of the biofilm structure on environmental conditions and the metabolic state of the population.

According to the results obtained, the absolute values of biofilm formation by *E. coli* and *E. faecalis* isolates corresponded to a low level of intensity. This is probably conditioned by the clinically healthy condition of the bird. Similar observations were described by C. Chiang-Ni *et al.* (2024), who established that non-clinical isolates of *Clostridium innocuum* most of often show a low ability to form biofilms, while pathogenic strains are characterised by a higher intensity.

E. coli of commensal origin have a significantly lower biofilm formation capacity compared to isolates obtained from animals or people with clinical pathology. In particular, R.P. Mahale *et al.* (2025) showed that biofilms formed only 16.6% of commensal isolates, while among clinical isolates – 77.2%. The study by D. Kalantar-Neyestanaki *et al.* (2023) demonstrated that in clinical strains, biofilm formation was accompanied by the presence of adhesion genes (icaADBC, mecA, fbe, etc.), whereas in non-clinical isolates, these genes were rare or showed low expression levels. However, even commensal isolates were able to form basic biofilm structures that ensure environmental sustainability and competitiveness in the microbial community. This is consistent with the data by K. Otokunefor *et al.* (2020), who showed the spread of signs of biofilm formation among natural populations of *E. coli*, not associated with pathology.

According to the results of bacteriological studies, it was found that chickens of both groups with different content were not found to have *Salmonella spp.*, *Listeria spp.*, and *Yersinia spp.*, which indicates that the bird was not contaminated with zoonotic pathogens at the time of the study. Moreover, differences in the structure of the commensal microbiota were found: in samples from free-range chickens, the microbial spectrum was mainly represented by *E. coli* and *Enterococcus spp.*, while in the vivarium conditions, *Klebsiella spp.* and *Pseudomonas aeruginosa*were additionally isolated, which may indicate the influence of a stable artificially controlled microecological environment on the preservation of opportunistic bacteria.

Comparison of the results obtained with the literature data shows that commensal isolates of *E. coli* and *E. faecalis* obtained from clinically healthy chickens, expectedly formed mainly low-density biofilms. This is consistent with the data by S. Ramos *et al*. (2020) and B.A. Lindstedt *et al*. (2018), who emphasised that most non-clinical strains of *E. coli* show only a basic level of adhesiveness and do not show the intense biofilm formation characteristic of pathogenic variants. Similarly, data by B. Krawczyk *et al.* (2021) and C. Geraldes *et al.* (2022) confirmed that *E. faecalis* commensal origin usually forms weak biofilm structures, while clinically significant strains have significantly higher adhesive activity.

In the current study, the average optical density values for *E. coli* and *E. faecalis* (0.264 and 0.217, respectively, in the free-range group) did not exceed the low-intensity limits, which is consistent with the characteristics of non-pathogenic isolates described by the above researchers. However, cultures obtained from free-range chickens had higher rates of biofilm formation compared to vivarium isolates. This is partially consistent with the data by T.T.M. Manders *et al*. (2025), who indicated that under more variable environmental conditions, bacteria are more active in implementing adaptive mechanisms, in particular biofilm formation. However, in the presented experiment, none of the isolates reached the levels of biofilm formation described in pathological strains, where the optical density values often exceed the threshold of medium or high intensity. This indicates that, despite certain differences between the keeping conditions, all isolates retained the commensal phenotype and did not exhibit properties characteristic of clinically significant variants of *E. coli* or *E. faecalis*. Thus, the data obtained in the current study are consistent with the literature reports on low levels of biofilm formation in non-clinical strains and simultaneously complement them, demonstrating that the intensity of this process can vary depending on the conditions of poultry keeping, but not to the level characteristic of pathogenic isolates.

Analysis of the intensity of biofilm formation showed that *E. coli*, so and *E. faecalis* formed mainly low-density biofilms, which correlates with the clinically healthy state of poultry and confirms the commensal status of isolates. However, bacteria isolated from free-range chickens had higher average levels of biofilm formation compared to vivarium isolates (by 41.17% for *E. coli* and by 58.4% for *E. faecalis*). These differences indicate the influence of environmental factors on the phenotypic realisation of biofilm properties**,** this is consistent with the data of leading international studies on the ecological adaptation of microorganisms (de la Fuente-Núñez *et al.*, 2013; Gupta *et al.*, 2016; Sonderholm *et al.*, 2017). Thus, it was found that the conditions of keeping poultry can modulate the biological properties of indicator components of the microbiome, in particular, their ability to form biofilms. The results showed the importance of taking into consideration environmental factors, depending on the conditions of keeping chickens.

Conclusions

In the conducted studies, the intensity of biofilm formation in isolates of indicatory bacteria isolated from chickens kept in various conditions was determined. *E. coli* and *E. faecalis* were selected as indicator microorganisms, given the available data on their possible synergistic interaction in the intestinal microbiome and their impact on the survival, development, and overall physiological condition of young poultry. In addition, it was noted that the coexistence of *E. coli* and *E. faecalis* can enhance the colonisation potential of both species and create conditions for chronic dysbiotic conditions.

It was found that isolates obtained from clinically healthy chickens generally formed low-density biofilms. This is consistent with the commensal status of the studied strains and confirms that high levels of biofilm formation are more often characteristic of pathogenic or colonising active bacterial variants. However, cultures isolated from free-range chickens showed a higher biofilm formation intensity compared to vivarium isolates: the average optical density for *E. coli* was higher by 41.17%, and for *E. faecalis* – by 58.4%. This may indicate that more variable environmental conditions (microbial diversity, diet differences, exposure to natural substrates) stimulate the activation of adhesion systems and the formation of biofilm structures as a survival mechanism for resident microorganisms.

These results support the hypothesis of ecological regulation of biofilm formation and indicate phenotypic plasticity of commensal isolates. However, to establish the exact mechanisms of adaptation, it is necessary to conduct molecular genetic analysis (assessment of the expression of genes encoding adhesion factors and matrix components), and transcriptomic and proteomic studies. Prospects for further research consist in expanding the range of microbiome species studied, analysing the relationship between biofilm formation and antibiotic resistance, and assessing potential risks to poultry systems in the context of the "One Health" concept.

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Conflict of Interest

None.

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Дослідження здатності до формування біоплівок *in-vitro* у санітарно-показових бактерій, виділених від курчат

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Анотація. У мікроорганізмів, що колонізують травний тракт тварин, зокрема домашньої птиці, біоплівки забезпечують стійкість до антимікробних засобів та імунної відповіді організму. Дослідження біоплівкоутворення індикаторних бактерій, ізольованих від курчат за різних умов утримання, дає можливість оцінити вплив екологічних факторів на їх фенотипову адаптацію та потенційний ризик для здоров'я тварин і людей. Мета роботи полягала у визначенні здатності до утворення біоплівок у бактерій *Escherichia coli* та *Enterococcus faecalis*, виділених від курчат, утримуваних у віварії та на вільному вигулі. Застосовано бактеріологічні, морфологічні, біохімічні та мікроскопічні методи досліджень. Інтенсивність біоплівкоутворення в індикаторних мікроорганізмів оцінювали за показником адсорбції/резорбції 0,1 % розчину кристалічного фіолетового з використанням полістиролових чашок Петрі. Оптичну щільність вимірювали спектрофотометрично при довжині хвилі 570 нм. Встановлено, що у зразках від курчат, що утримувались у віварії, виділено *E. coli*, *E. faecalis*, *Klebsiella spp*. та *Pseudomonas aeruginosa*, тоді як у курчат на вільному вигулі представників роду клебсієл та синьогнійної палички не виявлено, що

вказує на нижчу присутність потенційних патогенів у природних умовах утримання. Всі досліджені культури формували біоплівки низької або середньої щільності. Для ізолятів *E. coli*, отриманих від курчат вільного вигулу, середнє значення $\lambda = 0{,}264 \pm 0{,}09$, тоді як для ізолятів із віварію – $\lambda = 0{,}187 \pm 0{,}07$. Культури *E. faecalis* утворювали біоплівки з інтенсивністю $\lambda = 0{,}217 \pm 0{,}04$ у курчат на вільному вигулі та $\lambda = 0{,}137 \pm 0{,}03$ у курчат із віварію. Отже, ізоляти, отримані з природних умов, відрізнялися вищою інтенсивністю формування біоплівок – на 41,2 % (*E. coli*) та 58,4 % (*E. faecalis*) порівняно з умовами контрольованого мікроклімату. Це може свідчити про стимулюючий вплив факторів довкілля на експресію генів адгезії та біоплівкоутворення. Водночас усі досліджені культури були ізольовані від клінічно здорових птахів, що вказує на коменсальний характер мікробіому. Отримані результати є важливими для оцінки ризиків горизонтального перенесення генів стійкості та формування стабільних мікробних біоплівок у птахівництві

Ключові слова: індикаторні бактерії; *Escherichia coli*; *Enterococcus faecalis*; умови утримання птиці; фенотипова адаптація; санітарно-мікробіологічний моніторинг

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Quantitative changes in progesterone, estradiol and follicle-stimulating hormone in the blood serum of cows using hormonal preparations

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Abstract. Most modern enterprises producing animal products actively use hormonal preparations for animals without a detailed study of their effects on the body. This study aimed to investigate the impact of hormonal preparations on the dynamics of sex hormone levels in cows. The experimental groups of cows were formed according to the principle of analogous groups of 5 animals in each. The cows in experimental group 1 were administered 5 mL of the drug "Surfagon" intramuscularly, and the cows in experimental group 2 were administered 2.5 mL of the drug "Fertagil". The levels of follicle-stimulating hormone, progesterone and oestradiol were determined in the blood serum of cows during lactation on days 2, 6, 10, 14, 18, 22, 26 and 30. It was found that on day 2 of the study, the level of follicle-stimulating hormone in the blood serum of cows in experimental group 1 increased by 41.18% ($P < 0.05$), and in cows in experimental group 2, by 52.94% ($P < 0.01$) compared to the control. On days 26 and 30 of the study, the level of follicle-stimulating hormone in the blood serum of cows in experimental group 1 increased by 44-53% ($P < 0.05$), and in the blood serum of cows in experimental group 2 by 56-60% ($P < 0.05$). The progesterone content in the blood serum of cows in experimental group 1 remained unchanged until day 22. Then it increased by 18.22% ($P < 0.01$) in the experimental group 1, and by 15.42% ($P < 0.05$) in the blood serum of cows in experimental group 2, compared to the control. The level of oestradiol in the blood serum of cows in experimental group 1 increased by 1.79% ($P < 0.01$) from day 2 of the study and by 10.06% ($P < 0.01$) by the end of the study compared to the control group, which should contribute to better fertilisation of cows. The results obtained may be helpful for veterinary specialists and milk producers in regulating the effect of hormonal drugs on the bodies of cows and predicting the dynamics of their concentration in the blood

Keywords: cattle breeding; sex hormones; hormonal background; reproductive capacity; immunoenzymatic analysis

Introduction

The development of dairy farming depends on many factors, the most important of which are: obtaining high-quality milk, dynamic changes in herd size, and organising the reproductive capacity of the dairy herd at an economically viable cost. Numerous programmes have been developed for managing enterprise data, and one of the most progressive is the use of hormonal preparations for successful artificial insemination of cows. At the same time, it is essential to consider natural changes in the hormonal background of animals, as interference with the physiologically normal functioning of the humoral system can have adverse consequences for their organism.

E.M. Sitko *et al.* (2023) found that the use of dairy herd management programmes generally depends on the skilful organisation of the enterprise's economy and herd reproduction management. To assess this, the researchers used a combination of data, including the length of the waiting period, the success rate of herd fertilisation, the cost of feeding a specific herd, the length of gestation, and the efficiency of milk production. The authors note the importance of maintaining a herd reproduction programme, which is an integral part of the milk production cycle. At the same time, the best strategy is to organise artificial insemination of cows,

which largely depends on the use of appropriate hormonal drugs.

When administering various hormonal drugs to cows, several factors must be considered. As stated by N.A. Khudhair *et al.* (2021) note that one of the adverse effects of oxidative stress is its activation of the hypothalamic-adrenocortical axis, which in turn causes an increase in corticosteroids in the blood plasma of animals. This results in sharp changes in cortisol concentration, especially during pregnancy in cows, leading to an imbalance in reproductive hormones. Therefore, before using hormonal drugs, it is necessary to consider the possible risks to the cows' bodies. This is especially true at the beginning of the milk production cycle for young heifers. At the same time, as the authors emphasised, it is essential to adhere to the timing for first calving, which is achieved through skilful control of the hormonal background in the cows' bodies. The researchers found a pattern of interdependence between metabolic and sex hormones. By stabilising the concentration of sex hormones and successful artificial insemination, a significant shift in metabolic processes is possible. And when milk producers violate the procedure for artificial insemination of cows, several negative consequences for their bodies arise.

According to H.C. Evans *et al.* (2022), issues related to the organisation of dairy herd reproduction are linked to the functioning of the endocrine system. In particular, they are connected to hormone levels and their impact on both animal welfare and farm economics. The authors noted that hormone levels play a crucial role in regulating the homeostasis of cows, influencing their growth, development, and reproductive efficiency. The selected areas for improvement of these traits were based on the timely use of hormones, such as testosterone, estradiol, progesterone, and their synthetic analogues, including trenbolone acetate and zeranol, which can be strategically utilised in cattle breeding and meat production systems. At the same time, scientists have demonstrated that, despite significant advancements in the use of these drugs to regulate sex hormone levels in cows, the impact on the functional state of the cows' bodies, as well as on the human body that consumes products derived from such cows, remains uncertain.

After successful insemination of cows, the following critical stages are the period between three weeks before and after calving. M. Daudon *et al.* (2022) argued that it is during these periods that a sharp jump in metabolic processes and hormonal background occurs. The consumption of all necessary organic substances also increases actively, and metabolic adaptation systems are activated. In most cases, the energy requirements of the animal's body rise so much that they exceed the amount of energy compounds supplied by the feed. If a possible imbalance of nutrients in the animal's body is not prevented, a negative energy balance may develop. In this case, hormones that regulate homeostasis play an essential role. Therefore, according to the authors, correction of the hormonal background in cows, especially sex hormones, will help prevent the development of serious complications in the postpartum period.

In implementing a system for the successful development of cattle breeding in combination with reproductive technology development schemes, external risk factors may arise in addition to internal ones, as emphasised by W.S. Kim *et al.* (2023). These include, first and foremost, climate change. Sudden changes in temperature in the premises where animals are kept and, in the environment, can cause significant metabolic disorders in their bodies. As a result, stress develops in cows, which in turn provokes the release of cortisol, among other hormones. Therefore, the organisation of successful reproductive development in the herd

begins with stabilising the hormonal background of cows, taking into account the effects of external factors.

The use of various hormonal drugs to improve the reproductive capacity of cows in dairy and beef cattle farming is practically commonplace. At the same time, there is a wide variety of hormonal agents and methods for creating or extracting hormonal substances on the market. However, despite the active use of hormonal drugs and the success of cow fertilisation programmes, there are several problems. The introduction of hormones into an animal's body activates many mechanisms that are necessary to achieve the goal of their use, and, in turn, less attention is paid to the adverse effects on the hormonal balance of cows. After all, changes in hormone concentrations can cause disturbances in the animal's body homeostasis. At the same time, we must not forget about the risks to the human body that can be caused by consuming products obtained from such cows. Therefore, the study of changes in the hormonal background of sex hormones is a current and relevant issue today. The study aimed to establish the dynamics of sex hormone levels (follicle-stimulating hormone, progesterone and oestradiol) in the blood serum of Ukrainian black-and-white dairy cows after the administration of hormonal preparations before and during insemination.

Literature Review

Several factors can negatively impact the reproductive capacity of productive animals. The leading factors among them are environmental and technological factors, which, depending on their intensity of impact, can be both negative and conducive to the physiological reproduction of reproductive processes. As a result of their negative impact, animal productivity decreases, and pregnancy proceeds pathologically, which can lead to the birth of non-viable offspring. Therefore, to address several issues related to the reproductive capacity of animals, various schemes for using hormonal drugs to facilitate the successful insemination of cows have been implemented. However, problematic issues regarding the degree of influence of these substances on animal health remain open.

P.M. Fricke & M.C. Wiltbank (2022) investigated the effectiveness of the Ovsynch hormonal protocol for artificial insemination in cows. Thanks to the skilful use of appropriate hormone administration, the fertility rate of cows increased every year. The implementation of a multi-level hormonal background formation in animals contributed to the best preparation of cows for artificial insemination. However, it is not possible to clearly control changes in the hormonal background in cows, which leads only to a statistical effectiveness of fertilisation growth without taking into account the health status of the animals.

After studying the use of GnRH and PGF-2-alpha hormonal preparations, T. Afriani *et al.* (2025) established the dynamics of progesterone concentration in the blood of cows. According to their data, fluctuations in hormone concentrations were observed on the 11th day of the study, depending on the age of the animals. Older cows had higher progesterone levels than younger cows, indicating improved luteal phase function. However, artificial correction of the hormonal background in cows did not have a positive effect on the effectiveness of artificial insemination.

As confirmed by research conducted by Y.A. Amin *et al.* (2023), the use of the hormonal drug GnRH causes changes in sex hormones in cows. At the same time, an increase in the levels of progesterone, follicle-stimulating hormone and luteinising hormone was observed, as well as changes in testosterone and cortisol levels. The researchers also demonstrated changes in the concentration of minerals in the cows' bodies.

R. Mikuła *et al.* (2021) studied the condition of animals in the period after sharp hormonal changes. Spexin peptide hormone served as a marker in the study of changes in hormonal background, including progesterone and metabolites of sex hormones. Changes in hormonal levels and metabolic profiles in cows were observed between 21 days before calving and during the first 14 days of lactation. Along with changes in spexin peptide hormone levels, significant changes in hormone concentrations were observed, which affected metabolic processes in cows.

The postpartum period is crucial for both the physiological well-being of cows and the establishment of lactation processes. In their study, A. Sammad *et al.* (2022) concluded that timely insemination of cows had a positive effect on milk production technology. At the same time, emphasis was placed on the mandatory monitoring of hormone concentrations in cows during the postpartum period to reduce serious consequences for the body, namely the possible occurrence of a negative energy balance. The results of similar studies conducted by O.B. Pascottini *et al.* (2022) and R.P. Nevard *et al.* (2023) showed that the animal's body undergoes significant metabolic changes, particularly in various aspects of the body's immune defence, with the activation of adaptive mechanisms for the onset of the lactation period. This period is the most dangerous for the animal's health. If hormonal drugs are misused to improve the effectiveness of artificial insemination, they can lead to negative changes in energy balance.

Despite significant changes in cows' bodies that need to be taken into account when using hormonal drugs, there is a particular group of exogenous factors to which reproductive processes are sensitive. K. Miętkiewska *et al.* (2022) proved the importance of considering the impact of heat stress on the animal's body. According to the authors, sudden changes in climatic conditions pose a significant threat to the reproduction of cattle herds. It has been established that temperature fluctuations hurt the hormonal balance of cows. Therefore, taking this factor into account is also essential for effectively monitoring hormone levels in cows and improving the reproductive capacity of productive animals.

Materials and Methods

The study was conducted from 2022 to 2024 at Podilskyi Hospodar 2004 LLC in the village of Velyka Medvedivka, Shepetivka District, Khmelnytskyi Region, Ukraine on cows of the Ukrainian black-and-white dairy breed. All manipulations with cows were carried out in accordance with the basic principles of bioethics, in accordance with Article 26 of Law of Ukraine No. 3447-IV (2006), the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (1986); Directive 2010/63/EU (2010) and the General Ethical Principles of Animal Experiments adopted by the First National Congress on Bioethics (Law of Ukraine No. 249, 2012).

To conduct the study, three groups of animals were formed using the analogue method (one control group and two experimental groups), with five cows in each group. The cows in experimental group 1 were administered 5 mL of "Surfagone" intramuscularly 10-15 minutes before insemination. "Surfagon" stimulates the pituitary gland to release luteotropic hormone, which creates favourable conditions for fertilisation in the female genitals and stabilises the signs of oestrus in cows. Animals in experimental group 2 were administered 2.5 mL of "Fertagil" intramuscularly during artificial insemination. "Fertagil" contains gonadotropin-releasing hormone, which regulates the secretion of luteinising hormone (LH) and follicle-stimulating hormone (FSH)

and stimulates the growth and development of follicles, induces ovulation and controls the function of the corpus luteum.

Subsequently, a study was conducted to determine the levels of FSH, progesterone and estradiol during lactation on days 2, 6, 10, 14, 18, 22, 26 and 30. The principle of the method for determining follicle-stimulating hormone was based on the use of a two-site immunoenzymatic assay (DetectX® Progesterone ELISA, United States). A conjugate (second anti-FSH antibodies labelled with peroxidase) and the blood serum samples under investigation were added to the wells of a plate with immobilised antigen (specific anti-FSH antibodies). To obtain blood serum, the samples were incubated at 37°C in a thermostat. The clotted blood was separated from the walls of the tube using a glass rod. The serum was poured into centrifuge tubes and centrifuged for 10 minutes at 3,000 rpm. The resulting serum was then collected with a pipette. FSH from the sample binds to the antigen on the surface of the well and the conjugate. Material that did not bind was removed by washing. Next, the activity of the enzyme bound to the surface of the well in the plate became visible upon addition of the substrate. It was measured at a wavelength of 450 nm, along with the intensity of the colour reaction, which is directly proportional to the amount of FSH in the samples studied in pmol/L.

Progesterone and estradiol were determined using a test system based on competitive immunoassay. The test samples and conjugate (peroxidase-labelled progesterone/estradiol) were added to the wells of a plate with immobilised antigen (specific anti-progesterone antibodies). Progesterone from the test sample competed with the conjugate for binding to the antigen on the surface of the well. After washing, the activity of the enzyme bound to the surface of the well of the plate was revealed by the addition of a substrate and measured at a wavelength of 450 nm. The intensity of the colour reaction is inversely proportional to the concentration of progesterone in the test sample in pmol/L.

Statistical analysis of the study's results on sex hormone content in cow blood was performed using Microsoft Excel, with an assessment of the reliability of the difference in indicators and a Student's t-test analysis at significance levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$. Additionally, the mean values and standard deviations were calculated for each group and each study period. The normality of the data distribution was checked by analysing the variation characteristics. The results are presented as the mean ± standard error of the mean, which ensured the correctness of the comparison between groups.

Results and Discussion

After using the hormonal drug "Surfagon" in experimental group 1 and "Fertagil" in experimental group 2 before insemination, changes in the level of follicle-stimulating hormone in the blood serum were detected in cows. Changes in follicle-stimulating hormone were monitored for 30 days at 4-day intervals, reflecting changes in its synthesis process. The differences between the groups of animals that received hormonal drugs to stimulate fertilisation efficiency were compared with the control group (Table 1).

Table 1. Level of follicle-stimulating hormone in the blood serum of cows after administration of hormonal drugs, pmol/L (M ± m, n = 5)

Day of blood sampling	Control group	Experimental group 1	Research group 2
2	0.17 ± 0.02	0.24 ± 0.02	0.26 ± 0.01
6	0.15 ± 0.02	0.23 ± 0.01*8	0.25 ± 0.01**

Table 1. Continued

Day of blood sampling	Control group	Experimental group 1	Research group 2
10	0.13 ± 0.02	0.25 ± 0.02**	0.26 ± 0.02**
14	0.14 ± 0.02	0.23 ± 0.01	0.24 ± 0.01
18	0.08 ± 0.02	0.17 ± 0.02	0.18 ± 0.02**
22	0.13 ± 0.02	0.24 ± 0.01	0.25 ± 0.01**
26	0.15 ± 0.03	0.23 ± 0.02	0.24 ± 0.01
30	0.16 ± 0.03	0.23 ± 0.02	0.25 ± 0.01*

***Note**: *P < 0.05, **P < 0.01 compared to the control group*
***Source**: developed by the authors*

As can be seen from Table 1, on the second day of the study, an increase in FSH levels of 41.18% (P < 0.05) was observed in the blood of cows in experimental group 1, and an increase of 52.94% (P < 0.01) was observed in the blood of animals in experimental group 2 compared to the corresponding values in the control group. On the sixth day of the study, a higher concentration of FSH was observed. In the blood of cows that were administered "Sulfagone" intramuscularly, the FSH level increased by 53.33% (P < 0.05). In the blood of animals administered "Fertagil" intramuscularly, the concentration increased by 66.67% (P < 0.01) compared to the control. On the 10th day of the study, FSH levels in the blood of cows in experimental group 1 increased 1.92 times (P < 0.01), and in the blood of animals in experimental group 2, 2.0 times (P < 0.01) compared to the control group. Starting from the 14th day of the study, a gradual decrease in the differences in FSH levels was observed. In the blood serum of cows in experimental group 1, the FSH level increased by 64.29% (P < 0.05), and in the blood serum of animals in experimental group 2, it increased by 71.43% (P < 0.05). Despite a relatively sharp decline in hormone concentration on day 18 of the study, FSH levels in the blood serum of cows remained within acceptable limits. In the blood serum of cows that were administered "Sulfagone" intramuscularly, FSH levels increased 2.13 times (P < 0.01). In animals that received intramuscular injections of "Fertagil", the increase was 2.25 times (P < 0.01) compared to the corresponding values in the control group. On day 22 of the study, FSH levels in the blood serum of cows in experimental group 1 increased 1.85 times (P < 0.01), and in the blood serum of animals in experimental group 2, 1.92 times (P < 0.01) compared to the corresponding control.

In the following stages of the study, it was found that in the blood serum of cows administered "Sulfagone" intramuscularly, the FSH level increased by 44% on day 26 and by 53% on day 30 (P < 0.05). In contrast, the blood serum of animals that received intramuscular injections of "Fertagil" showed an increase of 56% and 60% (P < 0.05) compared to the control group. The analysis of the data revealed a general increase in the level of follicle-stimulating hormone, with a corresponding rise in its blood content in cows resulting from the additional use of hormonal drugs. The use of hormonal medications to increase the fertility of cows affected the concentration of progesterone. The experimental groups that received "Fertagil" and "Sulfagone" exhibited a gradual increase in the level of this hormone in their blood, as reflected in the biochemical analysis indicators. The characteristics of progesterone levels are shown in Table 2.

Table 2. Progesterone levels in the blood serum of cows after administration of hormonal drugs, pmol/L (M ± m, n = 5)

Day of blood sampling	Control group	Experimental group 1	Experimental group 2
2	0.35	0.36 ± 0.01	0.36 ± 0.01
6	0.34 ± 0.07	0.37 ± 0.01	0.35 ± 0.01
10	0.35 ± 0.01	0.36 ± 0.01	0.33 ± 0.01
14	0.33 ± 0.03	0.37 ± 0.01	0.32 ± 0.01
18	1.52 ± 0.1	1.65 ± 0.03	1.44 ± 0.05
22	2.14 ± 0.05	2.53 ± 0.07	2.47 ± 0.05
26	1.98 ± 0.07	2.32 ± 0.05	1.94 ± 0.09
30	2.82 ± 0.06	3.32 ± 0.08	3.43 ± 0.08**

***Note**: *P < 0.05, **P < 0.01 01 compared to the control group*
***Source**: developed by the authors*

Table 2 shows that from day 2 to day 18 of the study, only a tendency towards a change in the level of progesterone in the blood serum of cows was observed. Starting from day 22 of the study, it was found that the level of progesterone in the blood serum of cows in experimental group 1 increased by 18.22% (P < 0.01), and in animals in experimental group 2 by 15.42% (P < 0.05) compared to animals in the control group. On day 26 of the study, a significant increase in progesterone levels of 17.17% (P < 0.01) was observed in the blood serum of cows that were administered "Sulfagone" intramuscularly, compared to the control group. However, there were no statistically significant differences between the progesterone levels in the blood of cows administered "Fertagil" and the corresponding levels in the blood serum of cows in the control group. On day 30 of the study, the level of progesterone in the blood serum of cows in experimental group 1 increased by 17.73% (P < 0.01), and in the blood serum of cows in experimental group 2, by 21.63% (P < 0.01) compared to the corresponding values in animals in the control group. Changes in the level of the studied hormone are significantly manifested on the 22nd day of the study, with a notable increase observed in the experimental groups, reflecting the effect of these drugs on the processes of progesterone synthesis. The content of estradiol in the blood of cows, as determined by biochemical analysis of the control and experimental groups of animals during the study, exhibited a consistent pattern of increase in its level when using "Fertagil" and "Sulfagone". During the 30-day study period, the use of hormonal drugs stimulated higher levels of oestradiol, as shown in Table 3.

Table 3. Serum oestradiol levels in cows after administration of hormonal preparations, pmol/L (M ± m, n = 5)

Day of blood sampling	Control group	Experimental group 1	Experimental group 2
2	67.09	68.29 ± 0.11	67.48 ± 0.65
6	61.11 ± 0.46	67.26 ± 0.34	63.35 ± 0.37
10	61.45 ± 0.36	63.88 ± 0.52	63.70 ± 0.20
14	59.81 ± 0.62	63.71 ± 0.42	62.16 ± 0.34*
18	54.68 ± 0.75	62.32 ± 0.50***	54.68 ± 0.82

Table 3. Continued

Day of blood sampling	Control group	Experimental group 1	Experimental group 2
22	56.34 ± 0.25	57.80 ± 0.40	54.85 ± 1.06
26	58.64 ± 0.34	62.32 ± 0.52	62.23 ± 0.60**
30	45.30 ± 0.40	55.50 ± 0.68***	44.56 ± 1.26

***Note**: *P < 0.05, **P < 0.01 01 compared to the control group*
***Source**: developed by the authors*

As shown in Table 3, on day 2 of the study, the level of oestradiol in the blood serum of cows in experimental group 1 increased by 1.79% (P < 0.01) compared to the control group. In contrast, the level of oestradiol in the blood serum of cows in experimental group 2 did not show statistically significant differences compared to the corresponding control. On the sixth day of the study, the level of oestradiol in the blood serum of cows administered "Sulfagone" increased by 10.06% (P < 0.01). In the serum of animals administered "Fertagil", the concentration increased by 3.67% (P < 0.05) compared to the corresponding values in the control group. On day 10 of the study, a decrease in the level of oestradiol in the serum of cows was observed. At the same time, in the blood of cows in experimental group 1, the level of oestradiol increased by 3.95% (P < 0.05), and in the blood serum of animals in experimental group 2, by 3.66% (P < 0.05) compared to the control. On the 14th day of the study, the level of oestradiol in the blood serum of cows in experimental group 1 increased by 6.52% (P < 0.01), and in animals in experimental group 2, by 3.93% (P < 0.05) compared to the corresponding values in cows in the control group.

On days 18 and 22 of the study, the level of oestradiol in the blood serum of cows administered "Fertagil" intramuscularly, in both the control and experimental groups, did not show statistically significant differences. However, on day 18, the serum oestradiol levels in cows that received intramuscular injections of "Sulfagone" increased by 13.97% (P < 0.001), and on day 22 of the study, in the blood of cows in experimental group 1, it increased by 2.59% (P < 0.05) compared to the values in the control group. On day 26 of the study, an increase in the level of oestradiol in the blood serum of cows in the experimental groups was established. Thus, in the blood serum of cows in experimental group 1, the level of oestradiol increased by 6.28% (P < 0.01), and in the blood serum of cows in experimental group 2, by 6.12% (P < 0.01) compared to that in animals in the control group. On day 30 of the study, the estradiol level in the serum of cows that received intramuscular injections of "Fertagil" did not differ significantly from that in the control group. In contrast, in the blood serum of cows that were administered "Sulfagone" intramuscularly, the oestradiol level increased by 22.52% (P < 0.001). Thus, the study revealed a shorter time of "Fertahil"'s effect on stimulating oestradiol synthesis, which reflects the effectiveness of this drug.

Modern dairy farms always use reproductive hormones, and this is true for virtually every country in the world. In particular, according to M. Javed *et al.* (2023), the rapid development of artificial insemination and the popularisation of genetic material transport have led to a sharp increase in demand for this product worldwide. The use of pharmaceuticals is a necessary measure in modern conditions for the reproduction of cattle. In addition, with the increase in the world's population, the demand for high-quality and safe food products has grown, and the implementation of successful artificial insemination systems cannot

be achieved without the use of appropriate hormonal drugs. However, these substances also have adverse effects. If misused, they can lead to the loss of highly productive livestock, reduce the overall balance of hormones other than sex hormones, and generally cause homeostasis disorders. It is also worth noting that pharmaceuticals can remain in the bodies of animals in the form of residues, which can pose a danger to both animals and humans.

H.A. Sharawy *et al.* (2022) analysed a programme for the use of hormonal drugs called Ovsynch, assessing the effect of a synthetic analogue of sex hormones on reproductive performance and hormone levels. The experimental group of cows received intramuscular gonadotropin-releasing hormone at a dose of 10 μg according to the programme, and 7 days later, they received intramuscular synthetic analogue of prostaglandin F2α at a dose of 500 μg and intramuscular analogue of gonadotropin-releasing hormone at a dose of 10 μg, administered intramuscularly 48 hours after the administration of prostaglandin. An increase in the blood levels of progesterone and oestrogen was observed in the experimental group of cows compared to the control group. At the same time, cortisol levels decreased. When compared with the results obtained in this study, a similar trend was observed: with artificial stimulation of hormone synthesis processes, their total amount increased compared to the control group.

W. Barański *et al.* (2024) investigated the use of a gonadotropin-releasing hormone antagonist called Buserelin in cows to improve follicle-stimulating hormone release. Thanks to the controlled use of this pharmacological drug at a dose of 4 mcg, it was possible to increase the concentration of follicle-stimulating hormone in the blood of cows. As a result of this intervention, the ovulation process was successfully established. In the study, the effect of the administered drugs was assessed, and it was found that various changes in sex hormone levels occurred, depending on the pharmacological action of the administered drugs. This highlighted the need for a more detailed study of the effect of hormonal medicines on the body.

The popularity of prostaglandin and gonadotropin-releasing hormone preparations is due to their ability to activate reproductive capacity in animals. According to a study by A. Wicaksono *et al.* (2023), their use resulted in a reduction in the interval between calving and first insemination and an increase in the number of inseminations per cow. The use of these drugs also increased sex hormone levels. However, the authors emphasised that despite the positive results of the hormones used, the normal physiological reproductive capacity of cows was disrupted, and this required a more detailed study of the action of these agents and the appropriateness of their use. Considering the results of this work, different levels of hormones in the blood of the test animals were determined, which depended on the action of the administered hormonal drugs.

L.M. Vargas Ortiz *et al*. (2025) studied the effect of hormones applied in accordance with the J-Sinch and FTAI protocols. They found that the effectiveness of the hormonal drugs used was reflected in the level of sex hormones and affected the further functioning of the animals' bodies. At the same time, in cows from the experimental group treated according to the FTAI protocol, the levels of estradiol and progesterone in the blood increased. In contrast, the levels of follicle-stimulating hormone remained unchanged. In turn, D.Z. Bisinotto *et al.* (2024) investigated the effect of hormonal drugs on physiological processes in cows. It was established that the use of 17β-estradiol caused changes in the hormonal background in cows. At the same time, changes in hormone concentrations are more noticeable

when pharmacological drugs are used, as confirmed by studies.

As shown by the study by R. Sartori *et al*. (2024), the use of hormonal protocols based on progesterone and a combination of progesterone and estradiol causes changes in gonadotropic hormone concentration. The effectiveness of hormones was also assessed by fertility indicators, which depended on the concentration of sex hormones. The use of a combination of progesterone and oestradiol had a positive effect on the results of artificial insemination and ovulation induction. The use of progesterone alone in animal insemination protocols was less effective, which was caused by poorer stimulation of sex hormones in cows. W. Barański *et al*. (2024) evaluated the effect of the gonadotropin-releasing hormone agonist "Bruselin" on sex hormone levels in dairy cows. It was found that the use of this drug at a dose of 10-20 mcg caused an increase in the blood content of follicle-stimulating and luteinising hormones. The use of this hormone had a positive effect on the processes of the sexual cycle and the growth of follicles in the ovaries.

Analysis of the results of the current study and other scientific research indicates the effectiveness of hormonal drugs in dairy production. At the same time, for the best results, hormonal drugs are always used according to specific regimens. As a result, the duration of the effect of additional hormones on the body of cows varies from 5 to 40 days, depending on the specific technology used. As a result, the impact of these drugs on the body varies to a certain extent. The first time these hormones are used, the effectiveness of fertilisation will be pretty significant, as the level of sex hormones will be higher than in a regular sexual cycle. However, the next time, the effectiveness of fertilisation in cows that have been given the drugs will be lower because their bodies will become dependent on these injections. According to the results of biochemical blood analysis, this happens due to the intensive synthesis of sex hormones under the influence of artificial hormones. In general, such stimulation of the cows' bodies is not positive, as it disrupts the overall hormonal balance. Cases where the use of these drugs is necessary are the development of pathological conditions in the animal's body that make a normal sexual cycle impossible in production conditions. Therefore, it is advisable to use hormonal regimens in cases of fertility disorders, rather than in attempts to save materials on the farm.

Conclusions

This study focused on investigating the effect of hormonal drugs such as "Sulfagone" and "Fertagil" on sex hormone levels in cows. Changes in sex hormone levels in cows were observed when using two types of hormonal medications. It was determined that during the 30-day study, the level of hormones in the blood serum of cows varied depending on the drug used. When "Sulfagone" and "Fertagil" were administered to cows on the second day of the study, the level of follicle-stimulating hormone in the blood serum increased. Despite fluctuations in the level of this hormone, its concentration in the blood serum of cows remained higher than that of other hormones studied during the 30 days. When studying the level of progesterone in the blood serum of cows, it was found that there were no significant fluctuations in the concentration of this hormone from day 2 to day 18 of the study. However, starting from day 22, the level of progesterone in the blood serum of cows in experimental group 1 increased by 18.22% ($P < 0.01$), and in experimental group 2 by 15.42% ($P < 0.05$) compared to the corresponding control. In general, the serum of cows administered Sulfagone intramuscularly showed an increase in hormone levels compared to the control group until day 30 of the

study. At the same time, in the blood serum of cows treated with "Fertagil", hormone levels approached those in the control group by day 26 of the study, and from day 30 of the study, they increased again by 17.73% ($P < 0.01$). The level of estradiol decreased in the blood serum of cows in the control group during the 30-day study period, whereas in the experimental groups, it remained constant. When assessing the concentration of sex hormones in the blood of cows during the study period, it was found that the administration of the hormonal drug "Sulfagone" stabilised the concentration of progesterone and estradiol, in contrast to the use of the hormonal drug "Fertagil". In general, it has been demonstrated that the use of hormonal preparations results in an increase in hormone concentrations in the cows' bodies. In future studies, it is planned to evaluate the metabolic processes following the administration of these hormonal preparations to productive animals and to determine their effect on other body systems.

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Conflict of Interest

None.

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Кількісні зміни прогестерону, естрадіолу та фолікулостимулюючого гормону в сироватці крові корів за використання гормональних препаратів

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Анотація. Більшість сучасних підприємств із виробництва продуктів тваринного походження активно використовують гормональні препарати тваринам без детального вивчення особливостей їх впливу на організм. Метою дослідження було встановити, як гормональні препарати впливають на динаміку рівня статевих гормонів в організмі корів. Дослідні групи корів формувалися за принципом груп-аналогів по 5 тварин у кожній. Коровам дослідної групи 1 вводили внутрішньом'язово препарат «Сурфагон» у дозі 5 мл, а дослідної групи 2 – препарат «Фертагіл» у дозі 2,5 мл. У сироватці крові корів визначали рівень фолікулостимулюючого гормону, прогестерону та естрадіолу в період лактації на 2, 6, 10, 14, 18, 22, 26 та 30 добу. Встановлено, що на 2 добу дослідження у сироватці крові корів дослідної групи 1 рівень фолікулостимулюючого гормону зростав на 41,18 % ($P < 0,05$), а у корів дослідної групи 2 – на 52,94 % ($P < 0,01$) порівняно з контролем. На 26 та 30 добу дослідження рівень фолікулостимулюючого гормону у сироватці крові корів дослідної групи 1 зростав на 44–53 % ($P < 0,05$), а в сироватці крові корів дослідної групи 2 – на 56–60 % ($P < 0,05$). Вміст прогестерону в сироватці крові корів дослідної групи 1 не

змінювався до 22 доби, а в подальшому зростав на 18,22 % ($P < 0,01$), а в сироватці крові корів дослідної групи 2 – на 15,42 % ($P < 0,05$) порівняно з контролем. Рівень естрадіолу в сироватці крові корів дослідної групи 1, починаючи з 2 доби дослідження, підвищувався на 1,79 % ($P < 0,01$) і до кінця дослідження – на 10,06 % ($P < 0,01$) порівняно з контрольною групою, що має сприяти кращому заплідненню корів. Отримані результати можуть бути корисними для фахівців ветеринарної медицини та виробників молока щодо регуляції впливу гормональних препаратів на організм корів та можливості прогнозувати динаміку їх концентрації в крові

Ключові слова: скотарство; статеві гормони; гормональний фон; відтворюваність; імуноферментний аналіз

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Lymphatic effusions in cows: Diagnosis and treatment

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Abstract. The relevance of the study was determined by the insufficient study of the lymph changes in various pathological conditions of cows and the lack of effective approaches to lymphatic effusions diagnosis and treatment in veterinary practice. Thus, the aim of this scientific study was to improve the diagnosis of lymphatic effusions in cows and methods of their

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treatment. Clinical, planometric, cytological, haematological and statistical research methods were used in the study. The feasibility of using an environmentally friendly, natural remedy – a solution of Poltava bischofite – was substantiated. Clinical studies diagnosed similar lymphatic effusions in the area of the cow's withers, caused by injuries sustained due to design flaws in the farm's equipment. The development of lymphatic effusions was characterised by the formation of a weak inflammatory reaction, as evidenced by a relatively low number of leukocytes in the puncture. In smears from the lymph puncture, an increase in the number of dystrophically altered epithelial cells and leukocyte accumulation was noted. The therapy involved performing a relieving puncture, after which a 10.0% iodine solution (traditional method) was injected into the effusion cavity of some animals, and a solution of Poltava bischofite (proposed method) was injected into others. The clinical condition of the cows was monitored and the composition of the lymph effusion puncture was determined. A temporary exacerbation of the inflammatory reaction was noted after the introduction of the Poltava bischofite solution into the cavity, which was accompanied by an increase in the number of leukocytes and total protein content in the effusion puncture. During the treatment of cows with the Poltava bischofite solution, a decrease in the number of leukocytes in the blood was recorded, indicating its stimulating effect on the restoration of affected tissues. The number of erythrocytes and haemoglobin content in the blood of these cows also increased. The proposed method of therapy using Poltava bischofite solution has shown high therapeutic efficacy and can therefore be recommended as a therapeutic agent for traumatic effusions in cows. The results of the scientific study have practical value for veterinary doctors who ensure animal welfare

Keywords: cattle; animal welfare; closed mechanical injuries; lymph; Poltava bischofite

Introduction

The lymphatic system, as part of the cardiovascular system and immune defence, plays an extremely important role in the body. It provides drainage, metabolic and cleansing functions in the body, and is one of the first to respond to changes in tissues. Thus, its study is of great diagnostic importance in the work of a veterinary doctor. The lymphatic system consists of lymph, lymph nodes and lymphatic capillaries, vessels of various diameters, the lymphatic cistern and lymphatic ducts that flow into the bloodstream (cranial vena cava). Each of these links can be used to assess the condition of tissues in a specific area of the body.

Not enough attention has been paid to studying the lymphatic system in animals: more attention has been paid to lymph nodes (Febo *et al.*, 2023; Oblak *et al.*, 2024), and less to lymphatic vessels and lymph. According to H.P. Janardhan *et al.* (2023), there are still unanswered questions related to the layered arrangement of lymphatic vessels in organs and the composition of lymph. In particular, the following are considered drainage of cardiac lymph from all three layers of the heart from the subendocardium and myocardium to the subepicardium, and further to the mediastinal lymph nodes, and the heterogeneous origin of endothelial cells lining the cardiac lymphatic vessels; the integrated nature of lymphatic function or dysfunction in various diseases, including cardiac and renal diseases, and disorders of the structure and function of the intestinal lymphatic system and gastrointestinal

tract. Despite enormous progress in the structural description of lymphatic vessels, the issue requires further development.

In addition to the crucial role of lymphatic vessels in maintaining fluid balance in the body, in recent years, increasing attention has been paid to establishing the role of lymphatic vessels in organ-specific functions and immune surveillance. S. Arroz-Madeira *et al*. (2023) pointed out the importance of lymphatic vessels in maintaining tissue homeostasis and forming adaptive immune responses. In their work, B. Ponikowska *et al*. (2025) emphasised the role of the lymphatic system in the pathogenesis of internal diseases, which had previously been largely overlooked, and drew attention to the need to develop therapies, particularly for heart failure, that target the lymphatic system. Their studies tested various interventions, from mechanical removal of lymphatic stasis to pharmaceutical interactions and lymphatic microcirculation.

Y. Jian *et al*. (2025) pointed to the lack of effective methods for the prevention of lymphedema and devoted their research to finding them through the influence on the lymph nodes. Damage to the lymphatic system is an initiating factor in the development of a number of diseases, leading to the accumulation of lymph in tissue spaces, chronic inflammation with an imbalance of immunocompetent cells, fat tissue deposition and fibrosis, which further impairs the lymphatic system. The authors proposed the use of decellularised lymph nodes for effective regeneration of lymphatic vessels and their integration into the lymphatic system.

J.C. Lee & S.K. Lee (2024) observed and treated post-traumatic lymphatic effusions in the extremities. Chylous effusion in the wrist was treated with surgical incision and drainage, octreotide administration, and a low-fat diet. Two weeks after surgery, blood parameters returned to normal. S. Sotillo *et al*. (2025) presented the results of an analysis of fluids from various types of cavity effusions, including lymphatic effusions, in dogs and cats. They demonstrated that the leukocyte composition of effusions varies significantly depending on the aetiological factor and the pathogenetic mechanism of formation, in particular with a predominance of neutrophilic reactions in inflammatory processes and relative dominance of mononuclear cells in non-inflammatory conditions. This highlights the diagnostic value of cytological analysis of effusions and may be extrapolated to the assessment of lymphatic effusions in cattle with surgical pathology.

In general, lymph can be of particular diagnostic importance. It is a biological fluid formed by the combination of interstitial fluid with tissue metabolism products, apoptotic cells, cellular fluid, and circulating immune complexes. It plays a key role in every immunological process, including the maintenance of immunological tolerance, immunity to pathogens, autoimmunity, inflammation, and cancer. A review of the literature reveals the basis of the structural and functional organisation of the lymphatic system in normal and pathological conditions, but a number of questions remain unanswered. Currently, there was a lack of scientific information in the literature devoted to the study of lymph in cattle and its changes in various pathological conditions, as well as methods of treating these animals for lymphatic effusions. Although subcutaneous lymphatic effusions in cows are quite common on farms. Most of them are post-traumatic in nature. Closed injuries cause tissue separation and displacement and damage to blood vessels. Thanks to the coagulation system, blood vessels clot and bleeding stops, but lymph from damaged lymphatic vessels enters the space between tissues, where it accumulates and forms swelling. Considering the above, it remains relevant to study the composition

of lymph in cattle and methods of treatment for lymphatic effusions, which will affect the restoration of health and productivity in cows, ensuring their well-being. Thus, the aim of this work was to study lymphatic effusions in cattle and to search for new methods of treating sick animals using an environmentally friendly, natural remedy – a solution of Poltava bischofite.

Literature Review

For a long time, the composition of lymphatic fluid was virtually unknown. It was believed that the composition of lymph and blood was the same. L. Santambrogio (2018) noted that this lack of knowledge was largely due to technical difficulties in cannulating lymphatic vessels and the small amount of fluid collected, which hindered the study of lymph. Since 2010, progress has been made in understanding the mechanisms that regulate the formation, circulation, and composition of lymph. L. Weaver & B. Weaver (2025) emphasised that the lymphatic system of cattle is an integral part of the mechanisms that maintain homeostasis in the body, as well as immune responses and inflammatory reactions. Structurally, it originates from branched networks of lymphatic capillaries responsible for collecting interstitial fluid and transporting it through regional lymph nodes to central lymphatic vessels. K.C. Hansen *et al*. (2015) noted that interstitial fluid is a precursor to pre-nodal lymph, which is formed as an ultrafiltrate in the process of capillary microcirculation. Thus, most of the proteins contained in the blood are also present in the lymph. According to their data, the osmotic composition of lymph depends on the characteristics of plasma protein ultrafiltration. In addition, the process is influenced by proteins and molecules formed in tissues as a result of the metabolic and catabolic activity of each parenchymal organ from which lymph flows. The data obtained and new insights have led to a new understanding of the importance of the lymphatic system in the physiology and pathology of the body.

In their studies on mathematical models and cattle, C.M. Quick *et al.* (2014) noted that lymph flow is the main mechanism for returning interstitial fluid to the bloodstream and determined the functional responses of post-nodal mesenteric lymphatic vessels, which adapt to venous hypertension by reducing internal contractile activity. Lymphatic vessels have relatively thin walls and can collapse. They contain muscles that exhibit phasic and tonic contractions modulated by a number of vasoactive mediators. Increasing attention is being paid to the observation that lymphangions, segments of lymphatic vessels connected by valves, form units that function similarly to cardiac ventricles. Lymphangions can act as cyclically contracting chambers and are capable of actively pumping lymph against the axial pressure gradient. This property allows lymph to move from the low-pressure interstitial space to the higher-pressure venous system, as well as to respond to increases in interstitial fluid pressure by pumping more fluid out of the interstitium.

Pathological processes are considered to be accumulations of lymph, which are classified as pathological effusions. As noted by K. du Preez (2023), effusions are abnormal accumulations of fluid in the pleural, peritoneal, or pericardial cavities of the body, and they come in all shapes and sizes. Effusions fall into two broad categories: exudates and transudates. S.J. Quantrill & L. Dabal (2002), M. Tahara *et al*. (2011) emphasised the importance of establishing the aetiology of effusions and examining the cytology, protein, and lactate dehydrogenase in effusions to differentiate them. Microscopic examination is critical for determining the type of fluid and identifying specific cells or microorganisms that may cause

pathological changes. F.H. Alonso *et al*. (2022) found that excess fluid can accumulate in body cavities for many reasons, which vary in terms of the cytological properties of the effusion. For diagnosis, along with clinical examination methods, the number of cells and protein content must be determined. Microscopic examination is a critical aspect of the diagnostic procedure. It allows not only to fully classify the fluid, but also to identify specific types of cells or microorganisms that may be responsible for fluid accumulation. Thus, these data should always be interpreted together.

It is noteworthy that, according to research, fluid accumulation in organs is characteristic mainly of large animals. In particular, D. Funk & R. Neiger (2014) reported that in dogs and cats, the volume of fluid in the abdominal, pleural and pericardial cavities is quite small (usually less than 10 mL), and the fluid cannot be aspirated from them. Conversely, it accumulated in clinically healthy horses, cattle and camels. In addition, E. Monnet (2004) noted that chylothorax is rare in domestic animals and, as an exception, can be observed in cows.

The study of lymph, both in cases of excess accumulation and in normal conditions, is of great diagnostic importance, since lymph is an intercellular fluid and clearly reflects the state of processes occurring in cells, tissues and organs as a whole. It requires in-depth comprehensive analysis. In order to use lymph for diagnostic purposes, it was necessary to determine the characteristics of its composition in normal and pathological conditions, which became the task of further research. Thus, W.R. Hein *et al.* (1988) studied lymph from the ovaries or uterus at different stages of pregnancy in cows. They determined the lymph flow rate, cell composition, and levels of protein, progesterone, testosterone, estrone, etc. It was found that at all stages of pregnancy, the concentration of progesterone and androgens, in particular androstenedione, was higher in ovarian lymph than in uterine lymph or blood plasma. Lymph more accurately reflects the tissue cell environment than efferent blood, and further analysis of differences in the concentration of substances in lymph relative to their productivity in arterial and venous blood of the ovaries and uterus may lead to the identification of factors important for local regulatory mechanisms of the reproductive tract.

In their studies, C.M. Smuts *et al.* (2016) found that measuring lactate dehydrogenase activity may be useful for differentiating transudates and exudates in cats and dogs. Lactate dehydrogenase activity was significantly higher in exudates than in transudates and varied significantly depending on the measurement method. Studies of lymph composition are also aimed at assessing metabolic processes. In particular, J.L. Khol *et al.* (2012) evaluated the feasibility of collecting and examining lymph from the udder of cows to determine its diagnostic value. The results demonstrated the potential of such testing for the early detection of paratuberculosis in cows. Studies by a number of authors: K. Alitalo (2011), E. Weber *et al*. (2022) and K. Koltowska *et al.* (2023) pointed to the relevance of determining the composition of lymph and the clinical manifestations of its accumulation, as well as the condition of lymphatic vessels in diseases. Defects in lymphatic function can lead to lymph accumulation in tissues, weakened immune responses, accumulation of connective tissue and fat, and tissue oedema. The article highlighted how the lymphatic system contributes to the pathogenesis of various diseases, including immune and inflammatory reactions, and its role in the spread of tumour cells. Thus, based on studies of the lymphatic system, its key role in the pathogenesis of various diseases has been identified. Thus, the composition of lymph is an important diagnostic indicator that is significant for

determining the aetiology of pathological effusions and selecting treatment approaches.

Materials and Methods

The study was conducted over eight years (from 2018 to March 2025) at the "Trostyanets" dairy farm (Poltava region, Poltava district, Velykyi Trostyanets village). At the farm, a dispensary examination of approximately 300 cows of different ages and breeds was carried out in order to study the prevalence of lymphatic effusions. During this process, cases of diseases requiring surgical intervention were recorded, namely closed mechanical injuries of soft tissues.

During clinical examinations of cows with lymphatic effusions, attention was paid to the size of the swelling, the presence of signs of fluctuation, and the nature of the punctate. To determine the dimensions of the lymphatic effusions, a type 1 vernier calliper with a scale division of 1 mm was used. The volume of the effusions was calculated using the formula for a rectangular parallelepiped.

At the next stage, the contents of the swellings were examined. The morphological composition of blood was also determined using generally accepted methods in a Goryaev counting chamber (Ukraine). Comparisons were made between the values of haematological parameters in animals of the experimental groups diagnosed with lymphatic effusions and healthy cows of the control group. To determine the nature of the inflammatory process in affected cows, lymph samples were collected and smears were prepared. These were stained using the Romanowsky–Giemsa method. The smears were examined under a MICROmed XS-5520 microscope (Ukraine) and photographed using a Canon Power Shot III attachment (Japan).

The number of leukocytes in the lymph was counted in a Goryaev chamber (Ukraine) according to the following method: 0.4 mL of a 3% acetic acid solution with gentian violet (Turk's solution) was added to a test tube. The lymph in the tube was thoroughly mixed. Using a capillary pipette, 0.02 mL of lymph was drawn up, its tip was carefully wiped with a moistened and then dry cotton swab, transferred into the test tube and gently expelled. The pipette was rinsed several times with the diluting fluid, drawing it up to the level of the taken blood. The test tube was closed with a rubber stopper and left for 4 minutes, periodically mixing the contents. The lymph was then introduced under the ground glass of the Goryaev chamber. Leukocyte counting was started 1 minute after filling the chamber, when the lymph cells had settled. The number of leukocytes was counted at low magnification of the microscope in 100 large squares and multiplied by 50 to obtain the final result. The total protein content in the lymph was determined by the refractometric method (Refractometer RHC-300, China). Based on the refractive index value, the percentage of total protein was established using the Reiss table. Native blood from the animals was also examined; it was collected from the jugular vein and stabilised by the addition of heparin. In the blood of diseased and healthy cows, the number of leukocytes, erythrocytes and the haemoglobin content were determined using generally accepted classical methods.

After identifying the affected cows, treatment was carried out. For this purpose, 13 diseased cows were divided into two experimental groups with an even distribution according to the severity of lymphatic effusions. Animals of the first experimental group (n = 5) underwent a relieving puncture, after which a 10.0% iodine solution was introduced into the cavity (traditional therapy). If the lymph outflow did not stop, the procedure was repeated after 3 days. Treatment of cows in the second experimental group (n = 8) also involved a relieving puncture, after which a gauze drain impregnated with a solution of Poltava bischofite (Mineral LLC,

Ukraine) was inserted into the wound and left in place for 3 days. If the lymph outflow did not stop, the procedure was repeated. The control group consisted of clinically healthy animals (n = 5). Regular clinical examinations of the affected animals and monitoring of the effectiveness of the therapeutic agents were carried out over a period of 18 days.

The Poltava bischofite solution was selected as a treatment agent due to its properties. Bischofite is a natural mineral containing a large amount of magnesium chloride and other beneficial elements such as calcium, potassium, sulphates and others. Poltava bischofite is extracted from a great depth of up to 2.5 km, which ensures its environmental purity. The Poltava bischofite solution is used for therapeutic purposes in human and veterinary medicine, but in this work it was applied for the first time in cases of subcutaneous lymphatic effusions in cows.

Scientific studies involving animals complied with the requirements of the European Convention (1986) and the Law of Ukraine No. 3447-IV (2006). All necessary interventions on animals were carried out in accordance with the ARRIVE (n.d.) recommendations, without violating the guiding principles of Directive 2010/63/EU (2010). Statistical processing of planimetric, cytological and haematological data was performed using a personal computer with MS Office software, specifically Microsoft Excel. To determine the statistical significance of the obtained results, Student's t-test was used with significance levels of $P < 0.05$, $P < 0.01$ and $P < 0.001$. The results are presented as the mean value and the standard error of the mean ($M \pm m$).

Results and Discussion

The results of monitoring studies made it possible to assess the structure of surgical pathology in cows on the farm and determine the place of lymphatic effusions among other diseases requiring surgical intervention. It was found that the most common pathology remains hoof pathology – purulent pododermatitis and ulcers of the interdigital tissue, which accounted for about half of all other pathologies (Table 1). Less common were lymphatic effusions (lymph extravasations) in the withers area of cows and haematomas, and the least diagnosed were accidental purulent wounds.

Table 1. List of surgical pathologies in cows based on the results of a clinical examination

Pathology	Absolute number of cows, head	Relative number of cows, %
Inflammatory processes in the distal parts of the limbs	24	47.1
Lymphatic effusions	15	29.4
Haematomas	7	13.7
Wounds	5	9.8
Total:	51	100.0

***Source**: developed by the authors*

A clinical examination of the existing cow population helped to establish the prevalence of lymphatic effusions among animals kept on the farm. According to the results obtained, lymphatic effusions were found in 15 cows, of which 13 animals were diagnosed with the same type of lymphatic effusions in the withers area, accounting for 4.3% of the total herd. In the structure of surgical diseases, the number of cows with lymphatic effusions was 29.4%, which was second in prevalence after pathology in the distal part of the limbs.

During the course of the study, no seasonal pattern was observed in the occurrence of this disease. However, J. Jerlström *et al*. (2025) observed a seasonal increase in soft tissue injuries in cows. The researchers noted that traumatic injuries in cattle due to incidents on farms, animal transport, etc. indicated a decline in animal welfare. The study aimed to determine the prevalence and seasonal variations of traumatic injuries in cows and heifers raised on organic and conventional farms in Sweden. The results showed a higher prevalence in animals from conventional farms (9.8%) compared to organic farms (6.9%; $P < 0.001$).

The current study found that the appearance of lymphatic effusions in cows was caused by trauma and had the same location – in the area of the withers. Pathological changes varied in size. In some cases, the diameter was 8 cm, and in others, 15 cm. Based on the collected medical history, it was found that lymphatic effusions in the experimental cows occurred when the soft tissues in the withers area came into contact with a metal barrier pipe located along the entire length of the cowshed (Fig. 1).

Constant contact between the skin of the withers and the iron pipe caused chronic trauma in cows. As a result, the skin and subcutaneous loose tissue became detached. At the same time, small lymphatic vessels contained in the loose subcutaneous tissue were ruptured. As a result, lymph effusions under the skin were recorded, which was the main cause of local effusion formation.

A B

Figure 1. Chronic trauma as a cause of lymphatic effusions in cows
***Note**: A – position of the cow in the stall; B – cow's withers; arrows indicate swelling of lymphatic effusion in the cow's withers and a restrictive metal pipe*
***Source**: photo taken by the authors*

F.C. Furnaris & T.N. Constantin (2024) emphasised the need to optimise between two opposing elements: cow comfort and stall hygiene. The authors described two specific elements in the stall, namely: a chest locator (strap/tube) and a neck rail (strap/tube) – located above the stall partition, which minimise stall contamination. Only through structural optimisation can the welfare of cows on farms be ensured. S.F. Peek & T.J. Divers (2018) demonstrated that the neck area of cattle is at risk of lymphatic effusions due to the presence of numerous superficial and deep lymph nodes and, accordingly, a dense network of lymphatic vessels. Pathological conditions affecting these lymphatic structures often manifest clinically as non-inflammatory ("cold") or acute inflammatory ("hot") swellings, which varies significantly depending on the nature and duration of the underlying lesion.

The study found that lymphatic effusions in injured cows were characterised by typical

symptoms in the vast majority of cases. Thus, the animals developed undulating swelling in the area of the withers, which was clearly demarcated from the surrounding tissues. It was painless and the temperature in the damaged area remained unchanged. Upon examination of the pathological focus in five animals out of eight in the second experimental group, which accounted for 62.5% of cases, significantly pronounced local alopecia was established compared to the surrounding areas. The skin in these animals in the area of the withers had practically no hair cover and was thickened. When performing a test puncture of the swelling in the withers area in 6 animals (75.0%), lymphatic exudate (clear lymph was secreted) was noted, and in two patients (25.0%) – haemolymphatic exudate (lymph mixed with blood) (Fig. 2).

A B C

Figure 2. Lymphatic effusions
***Note**: A – local alopecia in the area of swelling; B – palpation examination of the pathological area; C – haemolymph*
***Source**: photo taken by the authors*

In 100% of cases, these were superficial lymphatic effusions. However, the size of the swellings in the affected animals varied. The symptoms of lymphatic effusions in animals in experimental group 1 did not differ significantly from those in experimental group 2, namely: a gradual slow increase in the size of the affected area of the body was observed. The exfoliated skin directly in the area of the pathological process thickened and had reduced elasticity. When attempting to palpate the affected area, the animal felt discomfort and tried to free itself from fixation, which indicated the presence of local pain. In chronic cases, in isolated cases, the formation of ulcers and the discharge of cloudy lymph were observed on the surface of the lymphoextravasate. The results of planimetric studies of lymphatic effusions in individual cows included in the 1st and 2nd experimental groups are presented in Table 2.

Table 2. Planimetric studies of lymphatic effusions in cows

Age, inventory number	Size of swellings, cm			V, cm³
	l	***b***	***h***	
5 years, 8751	10.5	9.5	8.5	847.8
6 years, 8719	15.0	11.0	12.0	1,980.0
5 years, 4952	13.0	8.0	9.0	936.0
6 years, 4953	8.5	11.0	9.5	888.2
7 years, 8568	11.0	22.5	6.5	1,608.0
6 years, 4230	12.5	10.7	7.5	1,003.0
5 years, 4361	10.5	10.0	9.0	945.0

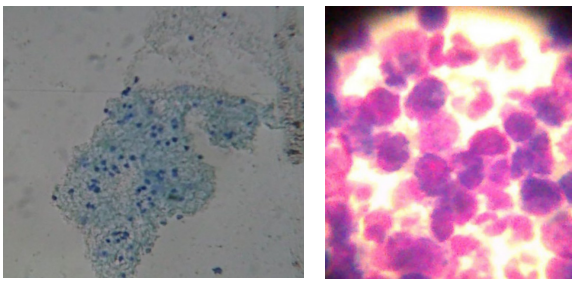
Table 2. Continued

Age, inventory number	Size of swellings, cm			V, cm^3
	l	*b*	*h*	
5 years, 4381	14.0	15.0	7.0	1,470.0
M ± m	118 ± 0.8	12.2 ± 0.9	8.6 ± 0.7	1,209.0 ± 150.5

***Note**: l – length, b – width, h – height, V – volume of swelling*
***Source**: developed by the authors*

Analysis of the data obtained showed that the volume of swellings in sick cows varied. Thus, the largest swelling size was 38.9% higher than the average value, and the smallest differed by 29.9%. The number of leukocytes was determined in the lymphatic effusions of sick animals from both experimental groups. It was found that the development of lymphatic effusions was characterised by the formation of a weak inflammatory reaction, as evidenced by the relatively low number of leukocytes in the puncture (5.5 ± 1.4 × 10^9/L).

When determining the total protein content in the lymph effusions, it was found that its average values did not differ significantly from the normative values in blood serum (72.0-86.0 g/L). Cytological studies of smears from lymph punctures of cows in both experimental groups revealed a significant number of epithelial cells. The latter were in a state of dystrophy and necrosis. A large number of different forms of lymphocytes were also found in smears from lymph puncture (Fig. 3).



A B

Figure 3. Cytological examination of lymph punctures from sick cows
***Note**: A – accumulation of epithelial cells with embedded lymphocytes; B – accumulation of lymphocytes*
***Source**: photo taken by the authors*

Thus, in cases of lymphatic effusions in cows, smears prepared from lymph punctates showed an increase in the number of epithelial cells, their dystrophic changes, as well as the appearance of foci with accumulations of leukocytes. In this context, the cellular composition in five microscopic fields of view was as follows: the number of lymphocytes was 144.3 ± 4.5, and epithelial cells 12.5 ± 1.1. N.V. Barbosa *et al.* (2024) reported similar data on the cytological composition of lymph in lymphatic effusions in small animals and emphasised the informativeness and necessity of conducting cytological examinations of lymphatic effusion punctates.

In accordance with the objectives set, studies of the morphological composition of blood were carried out. This included counting the total number of leukocytes and erythrocytes and determining the haemoglobin level (Table 3). In the blood of cows with lymphatic effusions,

compared with clinically healthy animals, a tendency towards an increase in the number of leukocytes by 35.6% was noted. A tendency towards a decrease in the number of erythrocytes in affected animals by 16.7% compared with the control was also established. At the same time, the haemoglobin content showed a tendency towards a slight decrease of 2.8% in the blood of cows in the first experimental group and by 8.0% in cows of the second experimental group.

Table 3. Morphological blood parameters of clinically healthy cows and cows with lymphatic effusions of the withers (M ± m, n = 5)

Indicator	Group of cows		
	"Control", clinically healthy animals	1st experimental, traditional therapy	2nd experimental, therapy with Poltava bischofite solution
Leukocytes, 10^9/L	10.1 ± 1.3	13.7 ± 0.1	13.8 ± 0.1
Erythrocytes, 10^{12}/L	6.0 ± 0.7	5.0 ± 0.1	5.0 ± 0.1
Haemoglobin, g/L	100.0 ± 0.8	97.2 ± 2.6	92.0 ± 3.6

***Note**: no significant difference was found between the values of the studied indicators in clinically healthy animals and those of the sick animals*
***Source**: photo taken by the authors*

The next stage of the work involved the treatment of the affected animals. Therapy of diseased cows included performing a relieving puncture of the injured withers area. After this, animals of the first experimental group received a 10.0% iodine solution into the cavity of the lymphatic effusion, whereas animals of the second experimental group were administered a Poltava bischofite solution in a volume of 40-60 mL. If lymph leakage did not cease, the procedure was repeated several times. It should be noted that on the third day of treatment in cows of the second experimental group, following the introduction of the Poltava bischofite solution into the cavity, a sharp change in the clinical picture was observed: the swelling became hot, painful and of a doughy consistency. At the same time, with the development of an inflammatory reaction provoked by the introduction of the Poltava bischofite solution into the pathological cavity, the number of leukocytes in the lymph increased by 42.1% and amounted to $9.5 \pm 1.2 \times 10^9$/L (Fig. 4).

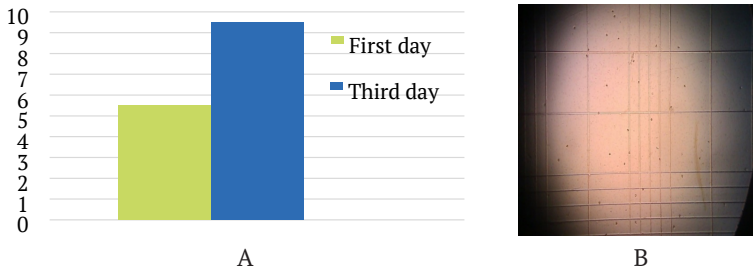


Figure 4. Determination of the number of leukocytes in lymphatic effusions obtained from cows in the second experimental group
***Note**: A – dynamics of leukocyte content in lymphatic effusions before and after the application of Poltava bischofite solution; B – microscope field of view for leukocyte counting*
***Source**: developed by the authors*

The increase in the number of leukocytes in the lymph of animals in the second experimental group during the development of an inflammatory reaction was combined with an 11.7-fold increase in the number of segmented neutrophils ($P < 0.001$) (Table 4). In addition, the appearance of band neutrophils and eosinophils was noted. At the same time, these processes occurred against the background of a decrease of more than 2.1 times ($P < 0.001$) in the number of lymphocytes compared to the first day of treatment of cows in the second experimental group.

Table 4. Leukogram of lymphatic effusions from sick cows in the second experimental group, % (M ± m, n = 3)

Study period	Indicator				
	E	N		M	L
		B	S		
First day	0	0	4 ± 0.9	1 ± 0.6	95 ± 1.2
Third day	1 ± 0.5	5 ± 0.3	47 ± 1.2***	1 ± 0.4	46 ± 1.6***

***Note**: *** – P < 0.001 compared to the baseline on the first day of treatment; E – eosinophils, N – neutrophils, B – band neutrophils, S – segmented neutrophils, M – monocytes, L – lymphocytes*
***Source**: developed by the authors*

An increase in the number of segmented and band forms of neutrophils in the punctates of lymphatic effusions of diseased cows in the second experimental group indicates the development of an inflammatory response. Activation of non-specific factors of the animals' resistance occurred, namely microphagocytic reactions. This is a consequence of the activating action of Poltava bischofite and confirms its effectiveness in stimulating cleansing and reparative processes in the area of the lymphatic effusion. The influence of the Poltava bischofite solution on the formation of immunological processes has been reported in previous studies by O.B. Kyrychko *et al*. (2021a; 2021b).

Determination of the total protein content in the punctates of lymphatic effusions was carried out in order to assess the nature of the effusion and the intensity of the local inflammatory response, since an increase in protein concentration in lymph indicates increased permeability of the vascular wall, activation of exudative processes, and the course of resorptive and reparative changes in the affected area under the influence of treatment. As shown by the results, indicative changes were already observed on the third day of treatment in animals of the second group when the Poltava bischofite solution was used. The dynamics of total protein content in lymph punctates of effusions in cows of the second experimental group are presented in Table 5.

Table 5. Dynamics of total protein content in lymph punctates of effusions in cows of the second experimental group, g/L (M ± m, n = 3)

Age and inventory number of the cow	Study period, days	
	first	third
6 years, 4953	81.0	162.0
7 years, 8568	76.0	154.0
6 years, 4230	85.0	145.0
M ± m	80.6 ± 3.8	153.0 ± 7.1***

***Note**: *** – P < 0.001, compared to the punctate on the first day of treatment*
***Source**: developed by the authors*

After the introduction of the Poltava bischofite solution, an increase in protein content by 89.8% ($P < 0.001$) was observed compared with the first day of treatment. This process indicates the development of an acute inflammatory response in the lymph of diseased cows in the second experimental group. Thus, the data demonstrate the effectiveness of using this agent at the initial stage of treatment. During puncture of the injured area, the lymph acquired a blood-tinged colour. The walls of the pathological cavity filled with lymphoextravasate became tense, and the volume of the contents decreased. After evacuation of the lymph from the cavities, given the high intensity of the inflammatory response, repeated administration of the Poltava bischofite solution was not performed. Continuous clinical monitoring of the affected animals was carried out for 18 days. Analysis of the observation results was performed to evaluate the effectiveness of the therapeutic agents (Table 6).

Table 6. Dynamics of clinical parameters in cows with lymphatic effusions after therapy (n = 13)

Indicator	Group of animals	Day of observation					
		3	6	9	12	15	18
Presence of lymphatic effusion	2 ex	±	±	±	-	-	-
	1 ex	±	±	-	-	-	-
Total affected, head	2 ex	8	8	6	4	1	1
	1 ex	5	5	4	2	1	-
Recovered, head	2 ex	-	-	2	4	7	7
	1 ex	-	-	1	3	4	5
Local inflammation	2 ex	+	+	±	±	-	-
	1 ex	+	+	+	+	±	±
Recovered, %	2 ex	-	-	25.0	50.0	75.0	75.0
	1 ex	-	-	20.0	40.0	80.0	100.0
Affected, %	2 ex	100.0	100.0	75.0	50.0	25.0	25.0
	1 ex	100.0	100.0	80.0	60.0	20.0	-

***Note**: 1 ex – first experimental group; 2 ex – second experimental group; + – symptoms strongly expressed; ± – symptoms weakly expressed; - – symptoms not expressed*
***Source**: developed by the authors*

During clinical examination on the sixth day, no increase in effusion size was observed in two animals of the experimental group. Palpation of these cows revealed thickening of the skin and deeper tissues. The tissues in the pathological area were hot and painful to the touch. Upon further examination of the pathological area, it was found that on the ninth day there was a decrease in the area of inflammatory oedema and the degree of tissue pain on palpation. However, the local body temperature in the pathologically altered area was higher compared to other areas.

Upon examination of the pathological focus on the 12th day of observation of the sick cows in the experimental group, no pain on palpation was recorded, the inflammatory reaction was not pronounced, and local thickening of the soft tissues was noted. A similar picture was observed in two other animals in the same group, in which lymph secretion and the intensity of the inflammatory reaction decreased starting on the ninth day. By the 12th day, the inflammatory process and lymph secretion had ceased. Thus, by the 12th day of observation, the proposed method was 50% effective.

With practically similar dynamics of clinical symptoms on the 15th and 18th days of observation, the proposed method ensured the

recovery of 75% of cows in the experimental group. At the same time, one animal was recognised as having an improvement in the course of the pathological process, which was manifested by a decrease in the intensity of the detected clinical signs. However, its complete recovery was not recorded. This was characterised by the fact that during a clinical examination, a decrease in lymph secretion was noted in the animal, the pathological area became denser, and a formation the size of an average fist was formed, similar to a mucous bursa. In addition, the connection with the skin was not observed on the entire surface of the pathological process; it was mobile on some areas of lymphatic effusion. That is, a separate pathological formation similar to a mucous bursa was created.

Analysis of the data obtained showed that in cows of the first experimental group, the traditional method of treatment provided 100% effectiveness within 18 days, namely: lymph secretion in most animals stopped on the 15th day. During the treatment process, after two administrations on the 9th day, one animal (20%) recovered. Accordingly, after three manipulations, two animals (40%) recovered on the 12th day, and after four treatments, four animals (80%) recovered on the 15th day. At the end of the observation period on the 18th day, no sick animals were found among the total number of cows under study. Treatment of such pathologies with iodine solution is a classic method, but it has a number of disadvantages. Iodine has anti-inflammatory and antiseptic properties. At the same time, it can cause local irritation and burns, and with excessive absorption, it can cause thyroid dysfunction. Thus, this study proposes an alternative treatment method using bischofite solution, which has proven to be highly effective.

The results of clinical observations were confirmed by haematological studies of cows in the first and second experimental groups, which showed significant differences and revealed the peculiarities of the proposed and traditional treatment methods. By studying the morphological indicators of blood in dynamics (Table 7), it was found that on the fifth day of treatment in the native blood of cows in the second experimental group, the number of leukocytes decreased by 21.0% ($P < 0.001$), on the 15th day – by 23.2% ($P < 0.001$) compared to the first day of treatment, and by 18.0% ($P < 0.01$) and 19.1% ($P < 0.01$), respectively, compared to the animals in the first experimental group. This indicates a milder effect of the Poltava bischofite solution, which exhibits anti-inflammatory properties and stimulates the regeneration of affected tissues.

Table 7. Dynamics of haematological parameters in cows during treatment (M ± m, n = 10)

Indicator	Treatment period					
	1		5		15	
	Group of animals		Group of animals		Group of animals	
	1 ex	2ex	1 ex	2 ex	1 ex	2 ex
Leukocytes, 10^9/L	13.7 ± 0.1	13.8 ± 0.1	13.3 ± 0.2	10.9 ± 0.4***/**	13.1 ± 0.1	10.6 ± 0.3***/**
Erythrocytes, 10^{12}/L	5.0 ± 0.1	5.0 ± 0.1	5.3 ± 0.2	6.2 ± 0.3*/	5.1 ± 0.1	6.7 ± 0.3**/**
Haemoglobin, g/L	97.2 ± 2.6	92.0 ± 3.6	97.4±1.9	112.0 ± 4.5*/*	95.2 ± 1.5	115.8 ± 4.5**/**

***Note**: 1 ex – first experimental group (traditional treatment); 2 ex – second experimental group (therapy with bischofite solution); ***/ – P < 0.001, **/ – P < 0.01, */ – P < 0.05 compared to the corresponding results on day 1 of treatment; /** – P < 0.01, /* – P < 0.05 compared to the results of similar indicators in cows of the first experimental group*

***Source**: developed by the authors*

Thus, on the 5th day of treatment, a significant increase in the number of erythrocytes in native blood was observed only in cows of the experimental group 2 – by 24.0% ($P < 0.05$) compared to the results of this indicator on the 1st day of treatment. On the 15th day of treatment, the number of erythrocytes in the native blood of animals in the second experimental group increased significantly by 34.0% ($P < 0.01$) compared to the results of this indicator on the 1st day of treatment and by 23.9% ($P < 0.01$) compared to the values of the corresponding indicator in cows of the first experimental group, indicating positive changes in the processes of erythropoiesis.

The method of treatment of cows in the second experimental group also contributed to an increase in the haemoglobin content in native blood, which already on the 5th day significantly increased by 21.7% ($P < 0.05$) compared to the values of this indicator on the 1st day of treatment and increased by 15.0% ($P < 0.05$) compared to the corresponding results in cows of the experimental group 1 under traditional therapy. On the 15th day of treatment, the haemoglobin content in the native blood of cows in the second experimental group increased by 25.9% ($P < 0.01$) compared to its values on the first day of treatment and by 21.6% ($P < 0.01$) compared to its level in animals in the first experimental group. In previous studies using Poltava bischofite solution for other pathologies, a significant increase in the number of erythrocytes and haemoglobin content in the blood of animals was also recorded. This effect on the organism may be due to the presence of iron and other minerals in the Poltava bischofite preparation, which belong to the haematopoietic group and are directly or indirectly involved in haematopoiesis.

The results obtained confirm the modern view of the lymphatic system as an active component of local regulatory, immune and reparative processes, rather than a passive pathway for the outflow of interstitial fluid. As noted by L. Santambrogio (2018) and K.C. Hansen *et al.* (2015), the composition of lymph largely reflects the metabolic and inflammatory state of the tissues from which it drains. In the study, this was manifested by characteristic changes in the cellular composition and protein content in lymphatic effusions in cows, confirming the diagnostic value of lymph analysis in traumatic soft tissue injuries. S. Sotillo *et al*. (2025) in their study of lymphatic and other cavity effusions in dogs and cats found that fluid characteristics and cytological composition are important for determining the aetiology of effusions. Variations in leukocyte composition, particularly neutrophils, may be associated with inflammatory processes, confirming the importance of cytological analysis for the diagnosis and identification of the causes of pathology, which may also apply to lymphatic effusions in cattle.

The weak inflammatory reaction found in most sick cows, characterised by a low white blood cell count in punctures and a predominance of lymphocytes and epithelial cells with signs of dystrophy, is consistent with the data of S.A. Center (2012), who described similar cytological features for chronic non-inflammatory or low-inflammatory processes. At the same time, the introduction of Poltava bischofite solution caused a controlled exacerbation of the local inflammatory reaction, which manifested itself in a significant increase in the number of leukocytes in the lymph, primarily due to segmented and stab neutrophils. Such changes in the leukogram correspond to the mechanisms described in the literature for the transition from inert effusion to the active phase of cleansing the pathological cavity (Barbosa *et al.*, 2024). This indicates the activation of microphagocytic reactions and the involvement of non-specific immune defence, which is a necessary condition for further reparative processes.

The positive dynamics of haematological parameters in cows treated with Poltava bischofite solution, in particular, a decrease in the number of leukocytes in native blood at later stages of treatment and an increase in the number of erythrocytes and haemoglobin content, is consistent with the data of O.B. Kyrychko *et al*. (2021a; 2021b) on the systemic action of bischofite components. Analysing the data of the study of clinical changes in the damaged area of the body due to lymphatic effusions in cows and the results of the study of their haematological parameters under different therapeutic approaches, it was proven that the proposed method of treatment using a bischofite solution, unlike the traditional method of therapy, ensured faster recovery of the affected tissues after the exacerbation of the inflammatory reaction. In addition, the number of erythrocytes and haemoglobin content in the native blood of these cows increased significantly, indicating a positive effect of the components of the bischofite solution on the erythroid activity of the red bone marrow. This allows to consider the proposed treatment method as milder and more physiological compared to traditional iodine therapy, since it provides short-term local activation of inflammation with subsequent accelerated tissue recovery and normalisation of the overall condition of the cows.

Conclusions

The study conducted comprehensive clinical, cytological, biochemical and haematological studies of lymphatic effusions in cows, and experimentally substantiated the effectiveness of using Poltava bischofite solution as a local treatment for this pathology. The studies were aimed at improving the diagnosis of lymphatic effusions in cows and a comparative assessment of traditional therapy and treatment using Poltava bischofite. The study found that the number of cows with traumatic lymphatic effusions accounted for 4.3% of the total number of cows on the dairy farm where the experiment was conducted. In the structure of surgical diseases, the incidence of animals with this pathology was 29.4%, which in terms of prevalence among other pathologies was second only to diseases of the distal limbs. It was found that in cows with lymphatic effusions, the number of epithelial cells with signs of dystrophy significantly increased in smear-imprints from lymph puncture, and foci with leukocyte accumulation appeared. At the same time, lymphocytes and epithelial cells predominated in the cellular composition. The development of lymphatic effusions was characterised by the formation of a weak inflammatory reaction, which was characterised by a small number of leukocytes in the punctures of lymphatic effusions. At the same time, in the case of an exacerbation of the inflammatory reaction provoked by the introduction of a solution of Poltava bischofite into the pathological cavity, their number in the lymph puncture increased by 42.1%, and the total protein content by 89.8%. In the native blood of cows with lymphatic effusions, different treatment methods showed a tendency to increase the number of leukocytes by 35.6%, decrease the number of erythrocytes by 16.7% and slightly decrease the haemoglobin level compared to clinically healthy cows. In addition, it was found that the number of leukocytes in the native blood of cows treated with bischofite solution decreased by 23.2% compared to their value on the first day of treatment and by 23.2% compared to the values of this indicator in animals undergoing traditional therapy. The milder effect of Poltava bischofite solution with anti-inflammatory properties on the pathologically altered area of the body was also confirmed, which led to faster recovery of the affected tissues. In the native blood of such cows, an increase in the number of erythrocytes by 34.0% and an increase in haemoglobin content by 25.9% were noted

compared to the values of these indicators on the first day of treatment, and an increase of 23.9% and 21.6%, respectively, compared to the results of traditional treatment. It has been proven that the proposed local therapy using a solution of Poltava bischofite in animals with lymphatic effusions in the withers area provided 25% effectiveness by day 9, 50% by day 12, and up to 75.0% effectiveness by day 15, which indicates its high therapeutic efficacy alongside traditional therapy.

Thus, the proposed treatment method using Poltava bischofite solution can be recommended for use in applied veterinary medicine for traumatic lymph effusions in cows. Taking into account the data obtained on the therapeutic properties of Poltava bischofite solution, further research into the use of Poltava bischofite solution in veterinary medicine and animal husbandry is promising. In particular, studying the mechanisms of its effect on the course of inflammatory and reparative processes in soft tissues, optimising the dosage and duration of use for various clinical forms of lymphatic effusions, as well as evaluating the effectiveness of bischofite use in other types of traumatic and postoperative complications in cattle and other animals.

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Conflict of Interest

None.

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Лімфатичні випоти в корів: діагностика та лікування

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Анотація. Актуальність дослідження обумовлена недостатнім вивченням змін лімфи корів за різних патологічних станів та відсутністю ефективних підходів до діагностики та лікування лімфатичних випотів у ветеринарній практиці. Тому мета цього наукового дослідження була спрямована на вдосконалення діагностики лімфатичних випотів у корів та методів їх лікування. У роботі використовувалися клінічні, планометричні, цитологічні, гематологічні та статистичні методи дослідження. Обґрунтовано доцільність застосування екологічно чистого, природного засобу – розчину полтавського бішофіту. Клінічними дослідженнями діагностовано однотипні лімфатичні випоти у ділянці схилу холки корів, спричинені травмами, отриманими через конструктивні недоліки обладнання ферми. Розвиток лімфатичних випотів характеризувався формуванням слабкої запальної реакції, свідченням чого була відносно низька кількість лейкоцитів у пунктаті. У мазках-відбитках із пунктату лімфи відмічалося збільшення кількості дистрофічно змінених епітеліальних клітин та скупчення лейкоцитів. Терапія передбачала проведення звільняючої пункції, після чого в порожнину випоту одним тваринам вводили 10,0 % розчин йоду (традиційний спосіб), а іншим – розчин полтавського бішофіту (запропонований спосіб). Спостерігали за клінічним станом корів та визначали склад пунктату з випоту лімфи.

Відмічали тимчасове загострення запальної реакції за введення в порожнину розчину полтавського бішофіту, яка супроводжувалась зростанням у пунктаті випоту кількості лейкоцитів та вмісту загального білка. У процесі лікування корів із застосуванням розчину полтавського бішофіту фіксували зменшення в крові кількості лейкоцитів, що свідчить про його стимулюючий вплив на відновлення уражених тканин. У крові цих корів також зростала кількість еритроцитів та вміст гемоглобіну. Запропонований спосіб терапії з використанням розчину полтавського бішофіту виявив високу лікувальну ефективність, а тому може бути рекомендований як терапевтичний засіб за травматичних випотів у корів. Результати наукового дослідження мають практичну цінність для лікарів ветеринарної медицини, які забезпечують добробут тварин

Ключові слова: велика рогата худоба; добробут тварин; закриті механічні ушкодження; лімфа; полтавський бішофіт

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The functional state of service dogs under the influence of strong stress factors

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Abstract. Service dogs are more stress-resistant than other animals, but they are also susceptible to stress factors, which can lead to changes in their bodies and their ability to perform their tasks. In this regard, the aim of the study was to investigate the effect of excessive stress factors

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in the form of powerful ballistic shelling of the territory on the organism of service dogs at the physiological and behavioural levels and to identify markers of stress disorders in these animals. Visual examinations, morphological, biochemical, computational and statistical methods were used to determine the main criteria that help differentiate stress markers in service dogs. It was found that physiological criteria, such as heart rate and breathing rate, and behavioural reactions change even before the shelling due to the hypersensitive hearing and increased sensitivity to vibrations in service dogs, and quickly return to normal within an hour after the stressor has ceased. At the same time, the enhanced response to stressors does not affect the ability of service dogs to perform their work tasks. It was also found that service dogs aged 4-5 years are more resistant to stress, as confirmed by the absence of significant changes in serum cortisol levels during the study. Service dogs aged 1-1.5 years are more susceptible to stress factors, as evidenced by a 6.68-fold increase in cortisol levels ($P < 0.001$) during the first stress factor and a 1.69-fold increase ($P < 0.001$) after repeated exposure. Studying the condition of service dogs under stress factors will allow the development of effective methods for diagnosing and preventing stress in animals and maintaining their working capacity. The results of the study can be useful for veterinarians in developing diagnostic criteria and methods for monitoring and correcting changes in the body of animals caused by stress

Keywords: acute stress; chronic stress; diagnosis; behavioural changes; haematological parameters; cortisol

Introduction

Service dogs perform tasks to ensure human safety, are used in operational and investigative activities, to detect prohibited substances or explosives, explosive devices, small arms and ammunition, in areas affected by natural disasters, perform working (mine detection) tasks in combat zones, as well as security or transportation functions, act as guide dogs or companion dogs accompanying people with sensory or mental disabilities. The issue of maintaining the working capacity of service dogs is particularly acute, as animals, like humans, are also subject to stress factors. The level of excitability, adaptability to stressors and inherited behavioural characteristics of the animal play a key role in the working capacity of a service dog. Scientists, dog trainers, and canine therapy specialists have been studying the development of service dogs' resistance to stress factors for a long time. Many studies have been conducted on the level of stress experienced by working dogs while performing their tasks. However, most publications concern therapy dogs and companion dogs.

Thanks to their developed sense of smell, dogs recognise the odours of various substances, including the hormones cortisol, adrenaline and noradrenaline, whose levels are particularly elevated during a stress response. As pointed out by L. Kiiroja *et al*. (2024), dogs are able to distinguish volatile organic compounds (based on adrenaline and noradrenaline) that are released during stress in the exhaled air of humans with high accuracy. Thanks to this unique ability, service dogs can be used to search for missing people, people trapped under rubble or avalanches. In addition, there is a whole field of application for dogs in medicine. In particular, C. Wilson *et al*. (2022) pointed out that dogs are used to detect hypoglycaemia, seizures,

dangerous bacteria, viruses (e.g. COVID-19) or parasites (e.g. malaria), epileptic seizures and even cancer in humans.

Service dogs play a special role in the rehabilitation of military personnel with post-traumatic stress disorder (PTSD). Military personnel who experience combat stress and prolonged traumatic effects require comprehensive treatment, including the use of therapy dogs for emotional support. As noted by S.C. Leighton *et al*. (2024), the use of therapy dogs helps to reduce the symptoms of PTSD in military personnel and improve their psychosocial functioning compared to conventional treatment practices. This is because assistance dogs have a unique mechanism for regulating stress indicators in veterans with PTSD by modulating the hypothalamic-pituitary-adrenal axis, which leads to the normalisation of cortisol (stress hormone) levels in the human body. In addition, as noted by L.O. Nieforth *et al*. (2024), positive interaction between dogs and humans leads to an increase in oxytocin (the happiness hormone) in both dogs and humans, which contributes to improved mood, reduced negative emotions, and a positive worldview. Studying the practice of using service dogs in the system of the State Emergency Service of Ukraine, H. Yatsenko (2022) noted that after 10-15 minutes of contact with a specially trained dog, a person experiences a reduction in the release of stress hormones at the neurovegetative level, normalisation of cardiac activity, decreased heart rate and pulmonary artery pressure, and an improvement in emotional state due to increased oxytocin levels, along with mobilisation of the body's internal resources.

At the same time, service dogs, like humans, are also exposed to stress factors, which may affect their performance of working tasks. Service dogs can also become fatigued, fall ill, experience low mood or sustain injuries. The cumulative triggering of stress factors may lead to refusal of the animal to perform its tasks or to atypical behavioural responses. It should be noted that dogs working in partnership with humans perceive the handler's emotional state and level of stress, which can also serve as an additional factor influencing the animal's performance. Stress resistance enables a service dog to respond appropriately to environmental conditions and stressors; however, even minor changes in the behaviour of a service dog must be detected in a timely manner and corrected.

The most common method used to assess stress in animals is the evaluation of behavioural responses and the analysis of cortisol samples in saliva, blood or hair. However, service dogs have specific characteristics compared with pet dogs, primarily due to their high stress resistance and adaptive capacity. E.A.E. van Houtert *et al*. (2023) studied dogs used for therapeutic purposes and found no significant differences in salivary cortisol levels, indicating the absence of an effect of work-related activity on physiological stress in these animals. At the same time, the number of publications assessing the physiological condition of search dogs or dogs working in combat zones remains limited. For example, in a study on the effects of sudden loud sounds on condition and behavioural responses, A.S. Mann *et al*. (2024) found that fireworks, thunder, gunshots and traffic noise are causative factors of increased stress in dogs. Fear of loud sounds of unknown origin, as demonstrated by S. Nichiporuk *et al*. (2023), is a primary aetiological factor in the development of PTSD in dogs during military operations. Stress experienced by service dogs can also have severe negative consequences, including loss of working capacity, which is often irreversible.

Given the lack of data in the literature on the impact of excessive stress factors in the form of intense and dangerous ballistic and cruise missile shelling on the nervous system

and stress development in service dogs, it is necessary to conduct a detailed analysis of the degree of adaptability of service dogs to such stress triggers, to determine their functional capabilities and, where possible, to predict their responses during the performance of their service duties. Thus, the aim of this study was to analyse and compare the physiological condition of service search dogs at rest and under the influence of severe stress factors, in particular ballistic missile shelling and/or presence in combat zones, and to identify stress markers in these animals.

Literature Review

Stress is a state in which an animal's body responds to endogenous and exogenous threats that help it to cope with danger or adapt to new conditions. The causes of stress in animals may include various physical, chemical and biological environmental stressors. The mechanisms underlying the development of stress responses and the organism's reactions to them have been extensively studied and described in the literature. In particular, depending on the intensity and type of the stressor, the rhythm of metabolic processes in the animal's body is disrupted, adaptive forces are mobilised and, due to increased activity of the endocrine organs, immune system function is reduced and catabolic processes are activated, leading to a decrease in humoral defence factors. The adaptive nature of the stress response often itself becomes a damaging mechanism that negatively affects overall resistance and immunological reactivity of the animal's body.

The role of the endocrine system in stress modulation is particularly important. In restoring homeostasis in response to a stressor, as noted by S.D. Clark *et al*. (2019), two endocrine subsystems are involved: the sympatho-adrenal medullary (SAM) axis, which acts through the catecholamines adrenaline and noradrenaline, and the hypothalamic-pituitary-adrenal (HPA) axis, which acts through increased levels of the glucocorticoids cortisol and corticosterone. T. Kooriyama and N. Ogata (2021) reported that the SAM axis responds to a stressor almost instantaneously: within milliseconds after exposure, the sympathetic nervous system activates the adrenal medulla to release adrenaline and noradrenaline. This leads to a sudden increase in the animal's energy demand, i.e. an immediate "fight-or-flight" response. Oxygen consumption increases through an accelerated respiratory rate; heart rate and blood glucose levels rise; locomotor activity, alertness, sensory and learning functions, and memory are enhanced. In contrast, processes that are not immediately essential for survival (digestion, reproduction and growth) are suppressed, and pain perception is reduced.

S.D. Clark *et al*. (2019) also noted that during prolonged exposure to a stressor, another mechanism of the stress response is activated: the adrenal cortex, following stimulation by the pituitary gland via adrenocorticotropic hormone (ACTH), begins to release glucocorticoids within several minutes or hours after exposure, with cortisol concentrations in plasma or saliva reaching a peak 10-30 minutes after cessation of the stressor. The main metabolic effect of cortisol is energy mobilisation, which initially helps to restore homeostasis. However, prolonged elevation of cortisol levels contributes to maladaptive changes in the organism, eventually leading to immunosuppression and delayed growth and development of the animal. Sustained elevation of cortisol suppresses glucose utilisation by cells and impairs the function of macrophages, neutrophils, basophils and eosinophils, resulting in reduced immune system function. In particular, according to T. Kooriyama & N. Ogata (2021), prolonged stress reduces immune system effectiveness by 40-70%.

Exposure to high-intensity stressors (for example, explosions), or sequential stress situations that do not allow animals sufficient time to recover, generally leads to behavioural changes. At the same time, as noted by N.J. Rooney *et al*. (2016), fear responses develop in animals when negative events exceed their individual tolerance threshold. Sensitisation (a process in which an animal's response intensifies with repeated stimulation) occurs more frequently when the stressor is of high intensity or low predictability.

At the same time, animals may also develop habituation both to stressors and to the working environment. In particular, in a study of service dogs, S.D. Clark *et al*. (2019) noted that salivary cortisol levels were lower in dogs that participated in several therapeutic sessions per week compared with dogs that worked once a week or less frequently. In addition, older dogs also showed a lower cortisol response than younger ones. Taken together, these results indicate that dogs are capable of habituating to their working environment and, as a result, exhibit a lower cortisol response to work-related situations compared with less experienced dogs.

As reported by L. Townsend & N.R. Gee (2021), prolonged exposure to stressors in dogs may lead to trigger-based stress accumulation. Thus, it is necessary to pay attention even to subtle signs of stress in service dogs, which are manifested through body language, such as yawning, lip licking, tongue flicking, gaze avoidance, a lowered tail, and ears held back. If these behavioural indicators are not recognised in a timely manner, the stress effect in the animal will intensify (a cumulative effect), which may ultimately result in increased aggression or depression, body tremors, excessive coprophagia, increased vocalisation, inappropriate behaviour, and loss of working capacity of the service animal. Consequently, implementing measures to reduce stress levels in service dogs is essential, as they require a balance between work, play and rest.

B.M.G. Gormally & L.M. Romero (2020) indicated that the development of stress within the animal's body is directly reflected by changes in glucocorticoid levels and the excretion of their metabolites in saliva, faeces and urine; alterations in heart rate, heart rate variability and respiratory rate; metabolic disturbances (metabolic rate, changes in thermoregulation); cellular impairments; changes in the immune system; delayed development; and behavioural alterations (including stereotypic behaviours). Thus, the most commonly used biomarkers for interpreting stress states in animals are plasma cortisol and glucose concentrations, substances involved in adrenal cortex responses. Although measuring glucocorticoid levels is not equivalent to measuring stress itself, because these hormones mediate the physiological stress response, they serve as physiological markers of stress in animals. In addition to cortisol, other markers can be used to assess acute stress in dogs, including adrenaline, noradrenaline and chromogranin A (CgA). The latter is released together with catecholamines during acute stress but is more stable.

N.L.B. Corder-Ramos *et al*. (2019) reported that many researchers prefer non-invasive methods, including the assessment of cortisol concentrations in hair and claws, as well as cortisol, catestatin and vasostatin in saliva. R. Palme (2019) indicated that measuring cortisol/corticosterone metabolites in faeces can also be used as a non-invasive method for assessing glucocorticoid release and adrenal activity. E.H. Kang *et al*. (2022) demonstrated that the measurement of salivary alpha-amylase can likewise be considered an important non-invasive method for assessing pain-related stress in dogs. At the same time, as noted by L. Mesarcova *et al*. (2017), determination of cortisol concentration in urine cannot be considered a

reliable indicator of stress in animals, since urinary cortisol reflects unbound, biologically active plasma cortisol, and measurement of free cortisol in urine has primary clinical significance in the diagnosis of Cushing's syndrome in dogs. Thus, the combination of behavioural changes and metabolic alterations in service dogs may indicate the development of cumulative stress, which in the future can lead to a decline in the working capacity of service dogs.

Materials and Methods

The study was conducted from October 2024 to June 2025 at the Department of Internal Diseases of the National University of Life and Environmental Sciences of Ukraine and at the Kyiv Region Canine Centre for service search dogs, within the framework of a research project carried out under contract No. BF/37-2021 dated 2 August 2021, "Scientific and practical foundations for ensuring animal health in Ukraine", in accordance with the task for 2025, "Effectiveness of natural-origin preparations in stress disorders caused by military actions in service dogs". Scientific research involving animals complied with the requirements of the European Convention for the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes (1986) and the Law of Ukraine No. 3447-IV (2006). All necessary procedures involving animals were carried out in accordance with the ARRIVE recommendations (Kilkenny *et al.*, 2010), without violating the guiding principles of Directive 2010/63/EU (2010) on the protection of animals used for scientific purposes.

The objects of the study were service search dogs from the canine centre. Fifteen clinically healthy dogs were examined and divided into three groups. At the first stage, under conditions of rest, a control group was examined consisting of five animals aged 4-5 years with a body weight of approximately 35.5 kg. At the second stage of the study, after exposure to excessive stress factors, five dogs from group 1 (German Shepherd Dogs and Belgian Shepherd Dogs, aged 4-5 years, with a body weight of approximately 35.5 kg) and five dogs from group 2 (German Shepherd crossbreeds aged 1-1.5 years, with a body weight of approximately 30 kg) were examined. The group of animals aged 1-1.5 years was added in order to differentiate whether age influences the manifestation of stress responses in service dogs.

The animals were housed at the service dog centre and were used for search work as intended. Dry feed Royal Canin Premium (France) was used for feeding. Observation of the animals was carried out periodically throughout the entire study period. Behavioural responses of the animals under resting conditions and under stress, as well as physiological indicators of functional status, were the subjects of observation. The animals were examined using generally accepted methods. The initial comprehensive examination included detailed history taking, assessment of general condition, visible mucous membranes, lymph nodes, the cardiovascular system, respiratory system, digestive system, urinary system and nervous system. Inspection, palpation, auscultation and laboratory blood tests were employed. To determine the functional state of the animals under the influence of excessive stress factors, a repeated examination was carried out. The stress factor consisted of intense, prolonged shelling of the territory of Ukraine with cruise and ballistic missiles by the aggressor. The repeated examination included assessment of body conformation, visible mucous membranes, lymph nodes, organs and systems, using inspection, palpation, auscultation and laboratory blood analysis.

Venous blood samples for analysis were collected from the superficial vein of the forearm in the morning before feeding. After shelling, blood sampling was performed within one hour after the end of the active phase of the attacks. For complete blood counts, 2 mL of

blood was collected into Vacumed tubes containing the anticoagulant K_3EDTA for morphological studies. For biochemical analysis, 2 mL of blood was collected into Vacumed tubes with a clot activator, then centrifuged for 10 minutes at 3,000 rpm, after which the serum was transferred into clean Eppendorf-type tubes.

Complete blood count tests were performed using a Mindray BC-5000 automatic haematology analyser (China). The following parameters were determined: haemoglobin content – by photometric method; number of erythrocytes, thrombocytes, leukocytes, absolute number of granulocytes, monocytes and lymphocytes – by electrical impedance method; haematocrit, mean corpuscular volume, mean haemoglobin concentration in erythrocytes, and haemoglobin content in erythrocytes were determined using a calculation method. The qualitative composition of erythrocytes and the differential composition of leukocytes were studied in blood smears stained with "Leukodif-200" haematological dyes manufactured by "Erba lachema" (Czech Republic). The ratio of neutrophils to lymphocytes (N/L) was calculated by dividing the total number of neutrophils by the total number of lymphocytes in absolute values, and the ratio of platelets to lymphocytes (Tr/L) was calculated by dividing the total number of platelets by the total number of lymphocytes in absolute values.

Biochemical blood tests of dogs were performed on a semi-automatic biochemical analyser LabLine-010 (Austria) using Spine Lab reagents (Granum, Ukraine). The serum was tested for total protein, albumin, glucose, urea, creatinine, total bilirubin, calcium, inorganic phosphorus, alkaline phosphatase activity, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyltransferase (GGT) were determined in the blood serum. During the biochemical study, standard methods were used: total protein content was studied by colorimetric method, based on the intensity of biuret complex formation, endpoint; albumin – by the bromocresol green reaction, end point; glucose – by the colorimetric method, enzymatic using oxidase, end point; ALT by a modified method of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), based on the rate of nicotinamide adenine dinucleotide (NADH) oxidation with measurement of absorbance decrease (kinetic); AST by a modified IFCC method based on the rate of NADH oxidation with measurement of absorbance decrease (kinetic); urea by the urease-glutamate dehydrogenase (GLDH) method (two-point); creatinine by a modified colourimetric Jaffé method (two-point); alkaline phosphatase by the p-NPP (p-nitrophenyl phosphate) kinetic method; calcium by the Arsenazo III method (end-point); and inorganic phosphorus by the phosphomolybdate reaction (end-point).

Serum samples (0.5 mL) were delivered within one hour to the veterinary laboratory "Bald" (Kyiv, Ukraine) for cortisol analysis. Serum cortisol concentration was determined using an ImmunoChem-2100 analyser (USA) by an enzyme-linked immunosorbent assay. The obtained results were processed statistically using Excel software, calculating the arithmetic mean, standard deviation and Student's t-test with significance levels of $P < 0.05$, $P < 0.01$ and $P < 0.001$, and comparing the probability of the animals' condition before and after shelling between groups of adult and young service dogs.

Results and Discussion

As a result of the examination of service search dogs at rest, it was established that at the beginning of the study, the physiological indicators corresponded to those of clinically healthy animals. Heart rate, respiration, and behavioural responses corresponded to the standards for service dogs (Levchenko *et al*., 2008). There were no visible signs of depression in the animals. The dogs were active, non-aggressive, and easily performed training exercises. All service

dogs, according to their purpose, underwent rigorous selection for breed characteristics and behavioural requirements, and possessed physical endurance, confidence, stress resistance, the ability to work in a team with humans, and a lack of fear of loud noises or aggression towards other animals and people. As a rule, dogs with average sensitivity were selected for work, as such dogs have balanced characteristics, are able to focus on the task and perform it. The morphological and biochemical blood parameters of the studied animals at rest also did not exceed the limit values, in accordance with the standards for healthy animals.

During the study, it was found that, thanks to their trained hearing, sense of smell and sense of vibration, service dogs began to get restless a few hours before the shelling, behaved excessively excitedly, ran around the enclosures, and exhibited increased vocalisation, which continued during the rocket fire. These symptoms were repeated during subsequent shelling. However, once the stressors had ceased, the service dogs quickly returned to their usual activities. After an hour, no signs of increased excitement or depression were observed in any of the animals; they were actively engaged in training exercises and ready to work. Evidently, engaging service dogs in constant physical activity and practising the necessary service skills allows service animals to reduce stress symptoms, as physical activity helps to release accumulated energy, reduce anxiety through the production of endorphins, and restore the animals' calm through interaction with the dog handler, which has a positive effect on the dog's well-being. The morphological indicators of service dogs' blood at rest and under stress also showed changes that characterise the animals' response to stress (Table 1).

Table 1. Morphological blood parameters of service dogs at rest and under the influence of stress factors (M ± m, n = 5)

Indicators	Control group	After severe stress factors, 24 days later		After repeated severe stress factors, 6 months later	
		Group 1	Group 2	Group 1	Group 2
Erythrocytes, 10^{12}/L	6.54 ± 0.93	8.06 ± 0.78	7.31 ± 0.48	7.59 ± 0.54	6.95 ± 0.50
Leukocytes, 10^9/L	7.92 ± 0.81	6.54 ± 0.85	9.24 ± 0.78•	8.45 ± 1.17	11.21 ± 1.35
Platelets, 10^9/L	105.33 ± 8.74	175.67 ± 27.18	108.05 ± 9.23•	212.25 ± 21.22*	179.0 ± 22.5**
Haemoglobin, g/L	154.75 ± 22.5	183.75 ± 18.21	162.35 ± 9.81	181.83 ± 11.89	163.67 ± 13.87
Haematocrit, %	52.15 ± 5.02	52.40 ± 8.0	48.10 ± 4.2	51.03 ± 3.2	47.32 ± 3.19
Neutrophils, 10^9/L	3.79 ± 0.42	4.92 ± 0.9	5.44 ± 0.36**	5.47 ± 0.55*	5.46 ± 0.04**
Lymphocytes, 10^9/L	2.76 ± 0.04	2.27 ± 0.61	1.99 ± 0.22	2.01 ± 0.16	3.17 ± 0.21
Monocytes, 10^9/L	0.58 ± 0.01	0.35 ± 0.03	0.84 ± 0.12*,••	0.38 ± 0.03***	0.57 ± 0.17
Eosinophils, 10^9/L	0.69 ± 0.01	0.56 ± 0.27	0.94 ± 0.32	0.54 ± 0.21	0.63 ± 0.26
Basophils, 10^9/L	0.01 ± 0.01	0.02 ± 0.01	0.03 ± 0.01*	0.01 ± 0.01	0.03 ± 0.01*

***Note**: * – P < 0.05, ** – P < 0.01, *** – P < 0.001 compared with the control group; • – P < 0.05, •• – P < 0.01, ••• – P < 0.05 compared between groups 1 and 2*
***Source**: developed by the authors*

As can be seen from Table 1, after stress exposure, the blood of animals in group 1 showed a 1.23-fold increase in the number of erythrocytes, a 1.67-fold increase in the number of thrombocytes, a 1.19-fold increase in haemoglobin content, and a 1.30-fold increase in neutrophils, while the number of leukocytes decreased by 1.21 times and monocytes by

1.66 times in absolute terms compared to the animals in the control group. However, no significant difference between the indicators was determined. In the blood of animals in group 2, there was a significant increase in the number of neutrophils by 1.44 times ($P < 0.01$), monocytes – by 1.45 times ($P < 0.05$), basophils – 3 times ($P < 0.05$), and a decrease in the number of lymphocytes by 1.39 times ($P < 0.01$) compared to the control group animals. In addition, there was a 1.41-fold increase in the number of leukocytes ($P < 0.05$), a 2.4-fold increase in the number of monocytes ($P < 0.01$), and a 3-fold increase in the number of basophils ($P < 0.05$) compared to group 1 animals. In a repeat study after exposure to a stress factor in service dogs, group 1 animals showed a 2.02-fold increase in the number of platelets ($P < 0.001$), a 1.44-fold increase in the number of neutrophils ($P < 0.05$), and decreased by 1.53 times ($P < 0.001$) compared to the control group. In the blood of animals in group 2, there was a 1.44-fold increase in the number of neutrophils ($P < 0.01$), a 3.0-fold increase in basophils ($P < 0.001$), and a 1.7-fold increase in platelets ($P < 0.001$) compared to the control group.

These data are consistent with N.L.B. Corder-Ramos *et al*. (2019), who indicated that during the development of acute stress in dogs, general leukocytosis developed, and a stress leukogram was recorded, characterised by neutrophilia, monocytosis, eosinopenia, and lymphocytopenia. The data obtained also consistent with the studies by E. Chmelíková *et al*. (2020), who noted that stress activates the hypothalamic-pituitary-adrenal axis, leading to the release of glucocorticoids and catecholamines, and then to an increase in the number of neutrophils and a decrease in the number of lymphocytes in the blood. In response to the action of glucocorticoids, circulating lymphocytes adhere to the endothelium of blood vessel walls and then migrate to other tissues, such as lymph nodes, spleen, bone marrow and skin, where they are sequestered. In addition, the release of glucocorticoids and catecholamines through cytokines promotes the production of acute phase proteins in hepatocytes, thereby increasing their levels in the blood serum.

The ratio of neutrophils to lymphocytes is also considered a marker of acute stress in animals. In service dogs, a change in the N/L ratio was observed after exposure to a stressor due to an increase in the number of neutrophils from 1.37 to 2.17 in dogs in group 1 and to 2.73 in dogs in group 2 during the first bombardment, and an increase in the N/L ratio to 2.72 in dogs in group 1 and to 1.72 in group 2 after repeated exposure to the stressor. These data are consistent with the results of a study by K. Radisavljević *et al*. (2015), who studied the effect of transport stress on dogs and found that the first physiological response is an increase in cortisol levels in blood plasma and saliva and an increase in the neutrophil/leukocyte ratio in the blood of animals. As noted by B.M.G. Gormally & L.M. Romero (2020), a change in the N/L ratio was recorded in the blood of animals 1-4 hours after exposure to the stress factor. In the bone marrow of dogs, the N/L ratio should be 1:1 or with a slight predominance of neutrophils, although variation is possible in adult animals. J.I. Cristóbal *et al*. (2022) established a range of reference values for the N/L ratio in healthy dogs, which was 0.74-5.62, and the platelet-to-lymphocyte ratio was 56.41-198.02.

The platelet-to-lymphocyte ratio (Tr/L) in dogs is an important indicator that reflects the presence of inflammation, infection, immune response and bleeding risk. An increased ratio may also be observed during the development of a stress response in the animal. In service dogs at rest, the Tr/L ratio was 38.2; under the influence of a severe stressor it was 77.4 in group 1 animals and 54.3 in group 2 animals. Following repeated exposure to the stressor, the ratio increased to 105.6 in dogs of group 1 and to 56.5 in animals

of Group 2. However, despite the considerable differences in results, the values of these indices in animals of all groups remained within the established reference ranges for healthy animals.

Biochemical parameters of blood serum (Table 2) in dogs of group 1 were characterised by a significant twofold increase in glucose concentration ($P < 0.001$) compared with the control group. In the serum of dogs of group 2, glucose concentration increased by 1.78 times ($P < 0.001$), inorganic phosphorus by 1.63 times ($P < 0.005$), while creatinine concentration decreased by 1.34 times ($P < 0.01$) and bilirubin by 1.83 times ($P < 0.001$). Repeated serum analysis in dogs of group 1 was characterised by a 1.15-fold increase in bilirubin concentration ($P < 0.01$) compared with the control group, whereas in group 2 only an increase in inorganic phosphorus concentration was observed, by 1.5 times ($P < 0.01$) compared with the control group and by 1.62 times ($P < 0.01$) compared with group 1.

Table 2. Biochemical indicators of blood serum in service dogs at rest and under stress factors (M ± m, n = 5)

Indicators	Control group	After severe stress factors, 24 days later		After repeated severe stress factors, 6 months later	
		Group 1	Group 2	Group 1	Group 2
Glucose, mmol/L	2.18 ± 0.10	4.36 ± 0.27***	3.90 ± 0.35***	2.15 ± 0.07	2.33 ± 0.31
Total protein, g/L	61.68 ± 3.53	53.06 ± 5.05	55.8 ± 4.02	59.38 ± 6.19	52.88 ± 3.77
Albumin, g/L	33.15 ± 3.29	31.58 ± 5.53	32.5 ± 2.35	27.75 ± 2.53	30.5 ± 4.59
Total bilirubin, μmol/L	2.75 ± 0.07	1.32 ± 0.36	1.50 ± 0.26***	3.15 ± 0.11**	2.94 ± 0.18
Urea, mmol/L	4.95 ± 0.78	5.67 ± 1.1	4.26 ± 0.65	4.35 ± 0.30	4.02 ± 0.48
Creatinine, μmol/L	94.88 ± 4.31	89.04 ± 15.03	70.80 ± 5.63**	91.03 ± 8.86	101.67 ± 9.5
Calcium, mmol/L	2.40 ± 0.40	2.66 ± 0.35	2.3 ± 0.36	2.26 ± 0.33	2.43 ± 0.17
Inorganic phosphorus, mmol/L	1.21 ± 0.10	1.76 ± 0.21	1.97 ± 0.32*	1.12 ± 0.16	1.81 ± 0.12**,●●

***Note**: * – P < 0.05, ** – P < 0.01, *** – P < 0.001 compared with the control group; ● – P < 0.05, ●● – P < 0.01, ●●● – P < 0.05 compared between group 1 and 2*
***Source**: developed by the authors*

During the study of serum enzyme activity in dogs of group 1, a 2.49-fold decrease in AST activity ($P < 0.05$) was observed compared with the control group, while in the serum of dogs of group 2 a 2.11-fold decrease in AST activity ($P < 0.01$) and a 2.17-fold increase in alkaline phosphatase activity ($P < 0.001$) were recorded compared with the control group (Table 3). Interpretation of AST and ALT enzyme activity in serum is used to assess liver function in animals and hepatocyte damage, while increased alkaline phosphatase activity has high sensitivity (86%) but low specificity (49%) for canine liver disease. However, during the development of a stress response, the activity of these enzymes may also increase due to physiological reactions such as muscle tension and oxidative stress. Investigating hepatic enzyme activity in service dogs, N. Hadžimusić & D. Hadžijunuzović-Alagić (2024) found that the age of the animals does not significantly affect liver enzyme activity, except for alkaline phosphatase, which is elevated in young animals due to bone growth.

Table 3. Enzyme activity in the blood serum of service dogs at rest and under stress factors (M ± m, n = 5)

Indicators	Control group	After severe stress factors, 24 days later		After repeated severe stress factors, 6 months later	
		Group 1	Group 2	Group 1	Group 2
ALT, U/L	42.60 ± 3.04	20.6 ± 4.53	36.45 ± 6.25	32.02 ± 2.99*	29.47 ± 4.78*
AST, U/L	45.15 ± 5.42	18.1 ± 1.98*	21.36 ± 3.69**	27.1 ± 5.77*	23.98 ± 2.84**
Alkaline phosphatase, U/L	97.13 ± 6.72	166.7 ± 11.94	211.2 ± 14.59***	106.0 ± 12.16	189.4 ± 26.45**,••
GGT, U/L	10.52 ± 2.70	12.57 ± 2.53	8.5 ± 0.15	5.4 ± 0.56	6.1 ± 0.12

***Note**: * – P < 0.05, ** – P < 0.01, *** – P < 0.001, compared with the control group; • – P < 0.05, •• – P < 0.01, ••• – P < 0.05 compared between groups 1 and 2*
***Source**: developed by the authors*

A repeated examination after 6 months also revealed a decrease in ALT activity by 1.33 times (P < 0.05) in the blood serum of dogs in group 1 and by 1.45 times (P < 0.05) in dogs in group 2 compared with the control group. At the same time, AST activity in the blood serum of dogs in group 1 decreased by 1.67 times (P < 0.05), while in dogs in group 2 it decreased by 1.88 times (P < 0.01). It was noted that AST activity at the beginning of the study was slightly higher than the reference values for dogs (5-25 U/L); therefore, the reduction in its activity in the blood of dogs from both groups is a positive finding, indicating normalisation of liver function in the animals. In addition, an increase in ALP activity by 1.95 times (P < 0.01) was observed in the blood serum of animals in group 2 compared with the control group and by 1.79 times (P < 0.01) compared with group 1.

The intensive use of service dogs and significant muscular loads may also contribute to increased activity of liver enzymes, especially AST, since part of this enzyme is present in muscle tissue. Manifestations of chronic stress in animals may likewise affect the intensive functioning of the liver, leading to changes in hepatocyte membrane permeability and the release of these enzymes into the bloodstream. At the same time, T. Ochi *et al*. (2013) indicated that alkaline phosphatase activity may be a useful biochemical marker of transport-related stress in dogs, as changes in its activity have been demonstrated in animals after transportation. The increase in alkaline phosphatase activity during stress is associated with elevated cortisol levels, which stimulate the production of the C-ALP isoenzyme in dogs in response to increased endogenous or exogenous corticosteroids.

A key marker of stress in animals is the hormone cortisol, a glucocorticoid produced by the adrenal cortex under the influence of ACTH. It regulates the majority of physiological processes in the animal body and, in the short term, mobilises energy and various body systems to respond to an immediate threat. However, prolonged elevation of cortisol levels often leads to impairment of the immune system and behavioural changes. In addition, cortisol regulates overall metabolism, stimulates myocardial contractility, increases arterial blood pressure and blood glucose levels, and exerts anti-inflammatory and immunosuppressive effects, helping the body to cope with inflammation, but at excessive levels it may suppress immune function. Excess cortisol can lead to the development of Cushing's syndrome in animals, increase the risk of heart failure, anxiety, aggression or depression, reduced appetite, provoke vomiting or diarrhoea, and cause skin disorders such as eczema, ulcers and inflammation. Excessively elevated blood glucose levels may result in the development of diabetes.

Reduced cortisol levels, in turn, may lead to weakened immunity, increasing susceptibility to infections and slowing wound healing. M. Siniscalchi *et al*. (2013) reported that cortisol has a significant influence on mood, behaviour and overall well-being in animals by regulating their responses to the environment. Thus, the determination of cortisol is considered a reliable biomarker for assessing the overall level of stress and the temperament of a dog.

E.A.E. van Houtert *et al*. (2023) found that in many publications authors focus on non-invasive sampling methods for determining cortisol concentrations in saliva, hair, and even in claws and milk. Cortisol levels in hair vary depending on seasonality, coat colour, age of the animal, and reflect the state of chronic stress in animals. D. Oyama *et al*. (2014) noted that salivary cortisol reflects increases in plasma cortisol with a delay of 20-30 minutes, while during prolonged exposure to a stressor its concentration in saliva decreases. However, it remains unclear whether this reduction represents adaptive physiological changes or habituation to new environmental conditions. I. Schöberl *et al*. (2017) reported that salivary cortisol levels reflect the activity of the hypothalamic-pituitary- cortisol may serve as a useful indicator of stress-coping mechanisms in dogs, as higher variability is associated with better regulation of the HPA axis and more adaptive stress coping, whereas lower variability may reflect blunted cortisol responses, potentially indicating chronic stress in animals.

E. Chmelíková *et al*. (2020) demonstrated that the determination of cortisol in saliva also has limitations related to the timing, method of sample collection, and storage conditions. N.J. Russell *et al*. (2007) established that storage of blood samples in freezers at –20°C does not significantly affect cortisol levels, whereas storage at +4-5°C reduces cortisol concentrations by approximately 12.5%. Thus, the assessment of any stress markers should be complemented by observations of behavioural changes in animals. It should also be noted that stress responses in animals do not always positively correlate with cortisol production and depend on the baseline excitability of the nervous system.

In the present study, considering the selected stress stimulus, it was appropriate to determine cortisol concentrations in blood. At the beginning of the experiment, this parameter was relatively high, amounting to 15.25 nmol/L (4.39 ng/mL) (Table 4). This value is slightly higher than those reported in the literature for blood cortisol levels in dogs or companion dogs. However, it is consistent with the findings of J. Wojtaś *et al*. (2020), who investigated search-and-rescue dogs involved in disaster zones, where the mean salivary cortisol concentration ranged from 4.2 to 4.89 ng/mL.

Table 4. Cortisol hormone content in the blood serum of service dogs at rest and under stress factors (M ± m, n = 5)

Indicators	Control group	After severe stress factors, 24 days later		After repeated severe stress factors, 6 months later	
		Group 1	Group 2	Group 1	Group 2
Cortisol, nmol/L	15.25 ± 2.3	21.82 ± 0.69	101.88 ± 8.21***,●●●	16.13 ± 1.85	25.77 ± 0.39***,●●●

***Note**: * – P < 0.05, ** – P < 0.01, *** – P < 0.001, compared with the control group; ● – P < 0.05, ●● – P < 0.01, ●●● – P < 0.05 compared between groups 1 and 2*
***Source**: developed by the authors*

As shown in Table 4, the cortisol concentration in the blood serum of dogs in group 1 increased 1.43-fold after the first mass missile strike and showed almost no difference after

the second, thus the results did not demonstrate a statistically significant difference. This may indicate that service dogs have a high level of stress resistance, which enables them to respond adequately to threats in dangerous situations. In contrast, in the blood serum of dogs from experimental group 2 a statistically significant increase in cortisol concentration was recorded, rising 6.68-fold ($P < 0.001$) after exposure to the first stress factor compared with the control group and 4.67-fold ($P < 0.001$) compared with dogs in group 1. This confirms the hypothesis that younger animals react more acutely to stressors and do not possess sufficiently developed stress resistance compared with adult service dogs. An increase in serum cortisol concentration in dogs of group 2 was also observed during the second mass missile strike. In particular, its level increased 1.69-fold ($P < 0.001$) compared with the control group and 1.6-fold ($P < 0.001$) compared with dogs of group 1 during this period of the study.

However, it is important to note that behavioural changes in dogs of group 2 did not reflect a stress response after the missile strikes. Despite the elevated serum cortisol concentration, the animals actively performed their tasks and interacted with their handlers. It is evident that the relationship with the human partner with whom the service dog works had an influence on the dog's physiological state. There is a hypothesis that reductions in cortisol levels in service dogs during stressful situations depend on dog-human interaction. T. Mitropoulos & A. Andrukonis (2025) pointed out that constant stress in the owner can lead to increased stress in service dogs. E. Chmelíková *et al*. (2020) indicated that the cortisol content in dogs' blood changes according to the circadian rhythm, with the highest peak observed in the morning after waking up and the lowest before bedtime.

A. Colussi *et al*. (2018) studied behavioral changes in dogs when they were played recordings of thunderstorms and thunder similar to the explosion of cruise missiles. Salivary cortisol concentrations in animals increased significantly 20 minutes after playback of the recording. The stress response to thunder sounds doubled salivary cortisol levels from a baseline of approximately 1.0 ng/mL to around 2.0 ng/mL. J. Wojtaś *et al.* (2020), studying service search dogs during tests simulating conditions after a natural disaster, found no increase in cortisol concentration in saliva. Elevated cortisol levels were not dependent on the dog's performance effectiveness or success during the examination. No stress-related behaviour was observed during therapeutic sessions either, indicating that the dogs did not experience excessive stress during the procedure. Thus, habituation through training is likely to help service dogs cope with excessive stress stimuli.

Investigation of changes in blood cortisol concentrations in service dogs can provide important insights into the animal's physiological health and behaviour, as well as improve understanding of how such dogs adapt to their environment and working tasks. Thus, monitoring clinical signs, as well as morphological and biochemical blood parameters in service dogs under conditions of increased stress load, is an essential component of the work of handlers and veterinary practitioners. The assessment of physiological and biochemical markers, such as cortisol levels and haematological and biochemical blood indices, enables effective evaluation of stress status in service dogs, contributes to understanding their adaptation to stressors and, when necessary, supports the maintenance of their functional capacity.

Conclusions

The present study described changes in the functional state of service dogs under the influence of excessive stress stimuli and identifies

markers reflecting the manifestation of stress responses in these animals. It was established that exposure to excessive stimuli, in particular intense mass shelling with ballistic and cruise missiles, leads to increased anxiety and vocalisation in service dogs before and during shelling. Physical activity and training sessions with a handler minimise stress-induced behavioural changes, which helps to maintain the working capacity of service dogs.

Service dogs aged 4-5 years are more stress-resistant, which is reflected in both behavioural reactions and the results of morphological and biochemical blood tests. The manifestation of a stress response in these animals is indicated by a 2.0-fold increase in blood glucose levels ($P < 0.001$), a 1.44-fold increase in the number of neutrophils ($P < 0.05$), a change in the neutrophil/leukocyte ratio due to an increase in the number of neutrophils from 1.37 to 2.17 after the first bombardment and to 2.72 after the second. This is a manifestation of a stress leukogram in animals. At the same time, the absence of a reliable correlation between the cortisol content in the blood of these dogs and the optimal indicators of biochemical blood tests indicate a high level of adaptability of their organism, which was also observed after the second study.

Young service dogs aged 1-1.5 years are more prone to stress reactions. In particular, there was a 1.44-fold increase in the number of neutrophils ($P < 0.01$), a change in the neutrophil/leukocyte ratio due to an increase in the number of neutrophils from 1.37 to 2.73 after the first bombardment, and to 1.73 after the second; glucose content – 1.78 times ($P < 0.001$), alkaline phosphatase activity – 2.17 times ($P < 0.001$), cortisol level – 6.68 times ($P < 0.001$) under the action of the first stress factor. The same changes were observed in the repeated examination of animals in group 2. A sharp increase in serum cortisol levels in animals of experimental group 2, aged 1-1.5 years, compared to animals of experimental group 1 during the first and second bombardments, indicates a stronger stressful effect of excessive stimuli on animals of group 2 and, accordingly, a more powerful physiological response of the organism to stress factors.

Further research should focus on identifying behavioural changes in service dogs directly involved in combat zones, the mechanisms underlying the development of post-traumatic stress disorders in these animals, and the development of methods for their correction. In the context of intense shelling of Ukrainian territory, it is promising to study more profound changes in the immune system of service dogs under the influence of stress factors, correlations between hormones, cytokines, and other poorly studied indicators that would allow for the timely diagnosis of the development of stress conditions in service dogs, contributing to an understanding of their bodies' adaptation to stress factors. It will also be important to analyse and scientifically substantiate methods of reducing the impact of stress factors on service dogs under intense stress, which will ensure the preservation of the service dog's working capacity.

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Conflict of Interest

None.

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Функціональний стан організму службових собак за впливу сильних стресових чинників

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Анотація. Службові собаки є стресостійкішими, ніж інші тварини, однак вони також піддаються впливу стресових чинників, що може призвести до змін в їхньому організмі та здатності виконувати поставлені завдання. У зв'язку з цим, метою роботи було дослідити вплив надмірних стресових чинників у вигляді потужних балістичних обстрілів території на організм службових собак на фізіологічному та поведінковому рівнях і визначити маркери стресових розладів у цих тварин. Візуальними дослідженнями, морфологічними, біохімічними, розрахунковими та статистичними методами було визначено основні критерії, які допомагають диференціювати маркери стресу в службових собак. Встановлено, що фізіологічні критерії, такі як частота серцевих скорочень і дихання, поведінкові реакції зазнають змін ще до обстрілу завдяки надчутливому слуху та підвищеному відчуттю вібрацій у службових собак і швидко повертаються до норми впродовж години після завершення дії стресора. При цьому, посилена реакція на дію стресових чинників не впливає на здатність службових собак виконувати свої робочі завдання. Виявлено також, що службові собаки віком 4–5 років є стійкішими до стресу, що підтверджено відсутністю достовірних змін вмісту кортизолу в сироватці крові впродовж дослідження. Службові

собаки віком 1–1,5 року сприйнятливіші до дії стресових чинників, що проявилося підвищеним вмістом кортизолу в 6,68 раза ($P < 0,001$) за дії першого стресового чинника та в 1,69 раза ($P < 0,001$) після повторної дії. Дослідження стану організму службових собак за дії стресових чинників дозволить розробити ефективні методи діагностики та профілактики стресових станів у тварин і збереження їх працездатності. Результати дослідження будуть корисними для лікарів ветеринарної медицини під час розробки діагностичних критеріїв та методів контролю і корекції змін в організмі тварин, викликаних стресовими явищами

Ключові слова: гострий стрес; хронічний стрес; діагностика; поведінкові зміни; гематологічні показники; кортизол

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Pathomorphological examination of dogs that died from gunshot wounds

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Abstract. The relevance of the study is due to the increase in the frequency of the use of firearms to kill animals during military conflicts. As evidenced by practice, most gunshot wounds are cases of animal cruelty. That is why a forensic veterinary expert must correctly assess not only the gunshot wound, but also the circumstances associated with the shooting. The purpose of this study was to identify and clarify the pathomorphological changes that occur in the organs and tissues of dogs in the area of a gunshot wound when shot with a firearm. As part of the forensic veterinary examination, a pathoanatomical autopsy was performed on two stray dogs that died from gunshot wounds. The first domestic dog received a penetrating wound to the abdominal organs during life, the cause of death of the second dog was a blind wound to the head. It was found that dystrophic and necrotic changes, and haemodynamic disorders, prevailed in the tissues of the gunshot wound. Focal and diffuse haemorrhages were recorded in skeletal muscle tissue, which were localised between stratified muscle fibres. Along with this, foci of

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haemorrhagic infiltration were noted. Massive thrombosis was detected in the lumen of large-calibre vessels, but the main changes were observed in the microcirculatory bed. Injuries that were recorded on the bodies of dead dogs were classified as serious bodily harm. They lead to pathological processes that are dangerous to the body and result in the death of the animal. The results obtained provide an opportunity to deepen the knowledge of forensic veterinary experts on the issues of animal damage by firearms, and the identified changes can serve to differentiate wounds caused by the action of such weapons from wounds of other origins

Keywords: forensic veterinary examination; entrance wound; exit wound; firearm; autopsy

Introduction

The spread of firearms, combat injuries, and uncontrolled shooting in the context of armed aggression and military operations on the territory of Ukraine has led to an increase in the number of wounded and killed domestic and stray animals, including dogs. Such cases often have signs of deliberate cruelty to animals, which requires a proper forensic veterinary assessment and evidence base for law enforcement agencies.

In contemporary scientific publications, considerable attention is paid to the development of forensic veterinary expertise of traumatic injuries in animals, especially in the context of countering ill-treatment and illegal use of weapons (Lemishevsky, 2024). The researcher emphasised the key role of pathomorphological research in establishing the mechanism of trauma, causes of death, and forming an evidence base for law enforcement agencies. The need for a systematic approach to the interpretation of injuries, considering morphological changes in tissues and the circumstances of the wound, which is of particular importance in armed conflicts, was highlighted. However, the available review works are mostly generalising in nature and do not always detail the pathomorphological features of gunshot wounds in dogs. Pathomorphological examination of gunshot injuries allows objectively establishing the mechanism of injury, the nature of the wound, the direction and distance of the shot, and the cause of death, which is key for documenting war crimes and countering animal cruelty in war conditions.

In the vast majority of cases, when animals are hit by firearms, soft tissues are primarily damaged, less often – parenchymal organs and the brain. However, the immediate cause of death is internal bleeding and pain shock. Visually, the damage will be accompanied by an entrance hole, which is a rounded defect up to 3 mm in size. The characteristics of the wound canal will depend on the morphological structure of the damaged tissue, on the degree of innervation and blood supply. If a parenchymal organ is affected, the entrance wound will have a star-shaped structure; if soft tissues are affected, haemorrhages will be present.

Clinical cases associated with injuries from the use of shells have a similar course to injuries from other types of weapons, so experts need certain markers for differential diagnosis. N. Bradley-Siemens & A.I. Brower (2016) described common types of shell injuries found in the United States of America, including firearms and ammunition associated with this form of injury. Three stages of ballistics exposure were discussed – internal, external, and terminal wounding.

R. Shrestha *et al*. (2023) detailed morphological features of gunshot wounds that are of

key importance for pathomorphological and forensic veterinary interpretation. Special attention was paid to the characteristics of the wound channel, which can be straight or tortuous, with zones of primary destruction, contusion, and distant tissue damage caused by the transfer of kinetic energy of the bullet. Secondary morphological changes were also described – bone fractures with radial cracks, ruptures of blood vessels and internal organs, massive haemorrhages, and with high-speed wounds – the phenomena of a temporary pulsating cavity. These signs are fundamentally important for pathomorphological examination of dog corpses, since they allow establishing the mechanism and direction of the shot, the approximate distance of the wound, and the immediate cause of death, which is important both for veterinary expertise and for the investigation of cases of animal cruelty.

J. Linder *et al.* (2023) devoted their research to the analysis of ballistic (firearm) injuries to the axial skeleton in 13 animals, mainly dogs. The researchers described the localisation of spinal and cranial injuries, neurological and ophthalmic manifestations, treatment approaches, and short-term clinical outcomes. T.H. Edwards *et al.* (2021) found that firearm injuries, as opposed to mechanical injuries, differ in terms of their mechanism of occurrence, clinical course, and various morphological manifestations. This fact is conditioned by the fact that gunshot injuries are a component of gunshot wounds and they will always complicate the pathogenetic situation.

K. Schrock *et al.* (2021) conducted a study of 97 animals with 137 acute fractures that were caused by gunshot wounds. Of these, 21 animals (15.3%) had fractures of the maxillofacial bone, 16 animals (11.7%) had spinal fractures, 8 animals (5.8%) had rib fractures, 56 animals (40.9%) had fractures of the distal long bones (below the knee and elbow joints), and 36 animals (26.3%) had fractures of the proximal long bones. Gunshot fractures generally have a high probability of poor outcome, which is associated with complications from soft tissue damage.

H. Baruah *et al.* (2021) found that with a gunshot wound, there will necessarily be an anatomical defect and functional disorders in the surrounding tissues, possibly bacterial contamination of the wound edges. The degree of damage to the tissue elements will depend on the ballistic characteristics of the projectiles: on the calibre, on the speed, on the shape and size, on the angle at which it enters the target, and on the anatomical structure and condition of the tissues at the wound site. However, the amount of energy transferred to the tissues will be crucial.

Given the small amount of literature data on the use of radiological imaging techniques in forensic veterinary medicine, M. Grela *et al.* (2021) attempted to evaluate the benefits of radiography and computed tomography (CT) in post-mortem diagnosis of gunshot wounds compared to classical autopsy. The experiment consisted of the following: 13 corpses of dogs were examined, in which the injuries were inflicted from different distances (1.5 and 12 metres) and one animal had a contact shot to the head. In each case, different types of ammunition were used, followed by X-rays and CT scans to investigate injuries sustained as a result of the shot. Post-traumatic bone damage and the presence of metallic foreign objects were successfully imaged in the study material using both X-ray and CT. GSW analysis using CT scans provided much better data quality and some additional information. Based on the results of experiments, the following general conclusions can be drawn. Firstly, the damage caused by a firearm correlates with the calibre, kinetic energy of the bullet, and the distance from the muzzle to the object of the shot is also very important. Secondly, radiological examination is useful for preparing forensic veterinary reports. They are

used as an adjunct to classical autopsies, and X-rays and CT scans increase the possibility of a more accurate post-mortem diagnosis.

The studied literature does not sufficiently cover the injuries that can occur under certain circumstances as a result of the use of firearms. In a few papers devoted to this problem, only macroscopic manifestations of the action of damaging factors of shots were studied and, as a rule, only the possibility of mechanical injuries (bruises, abrasions, wounds, bone fractures, damage to internal organs, and other anatomical structures) was stated. The available data were often unsystematic and were based solely on visual observations.

Each case of injury should be considered as individual and unique. Each gunshot wound is an injury that requires an individual approach – the same weapon and ammunition can cause completely different injuries, and different types of weapons and ammunition can cause the same or similar injuries. A veterinary physician acting as a forensic expert in the assessment of gunshot wounds in animals should have broad, unbiased knowledge and be able to analyse each case individually, without resorting to generalisations. Therefore, the purpose of this study was to determine the characteristic pathomorphological changes in the area of a gunshot wound in dogs injured by firearms.

Materials and Methods

The research was conducted from January 2024 to June 2025 at the scientific laboratory of the Department of Vertebrate Biomorphology named after Academician V.G. Kasianenko at the National University of Life and Environmental Sciences of Ukraine and at the Department of Normal and Pathological Morphology and Forensic Veterinary Medicine of Odesa State Agrarian University, within the framework of the interdepartmental research topic "Pathogenesis and Pathomorphology of Violent Death in the Context of Forensic Veterinary Examination", state registration No. 0123U102492. Live animals did not participate in the study. The authors considered the recommendations of ARRIVE (n.d.), however, since the present study was post mortem, it was based on OIE/WOAH standards (n.d.).

The study was conducted as part of a pre-trial investigation of criminal proceedings entered in the Unified Register of Pre-trial Investigations on the grounds of a criminal offence under Part 1 of Article 299 of the Criminal Code of Ukraine. The study material was the corpses of two mixed-breed dogs aged 4 and 5 years, who died as a result of gunshot wounds. Pathoanatomical examination of corpses was carried out in accordance with generally accepted forensic veterinary methods in compliance with the principles of completeness and objectivity of the examination. The autopsy was performed in the dorsal position using the complete evisceration method. Before starting the autopsy, a visual examination of animal corpses was performed, the general condition of the body, the presence of injuries, fatness were assessed, and attention was paid to the condition of the skin and mucous membranes of the respiratory tract, oral cavity, and eyes. After the autopsy, a sequential study of the subcutaneous base, muscles, bone system, thoracic and abdominal organs, central nervous system, and vascular bed was performed. Special attention was paid to the identification, localisation, and morphological characteristics of entrance and exit gunshot wounds, the direction and features of the wound canal, the presence of concomitant injuries and secondary pathological changes. Macroscopic assessment of injuries was performed taking into consideration the shape, size, condition of the wound edges, the presence of haemorrhages, tissue detritus, deformation of organs and bones. Signs of lifetime trauma were recorded, in particular, tissue reactions, vascular changes, haemorrhagic events, and manifestations of aspiration complications. All significant

morphological changes were documented by photographing. All data was carefully described and recorded in the protocol.

To investigate the morphological picture of injuries that occur during the use of firearms, histological examination of tissue micro-preparations from the sites of incoming gunshot wounds was performed. A key role in forensic veterinary examination was played by histomorphological changes that occur in the tissues that form the wound channel after a firearm shot. The study of these changes is important because it helps to clearly distinguish injuries caused by firearms from injuries of other origin (for example, caused by blunt or sharp objects, or shots from gas weapons), thus increasing the objectivity of the expert opinion.

For histological examination, tissue samples were taken from the areas of incoming and outgoing gunshot wounds, wound canal, adjacent soft tissues, and internal organs that were damaged. Subsequently, the selected material was fixed in a 10% solution of neutral formalin (pH 72-74). Haematoxylin and eosin were used for staining histological sections. Microstructural changes and histological structure in the preparations were studied under an MC 100 LED light microscope (Micros Austria). During the histological assessment, the nature of alterative, necrodystrophic and vascular changes, the presence of haemorrhages, thrombosis, destruction of muscle fibres, and the localisation and distribution of powder combustion products (soot, microparticles) in the structures of the skin and subcutaneous base were analysed. Interpretation of the obtained morphological data was carried out based on literature sources on forensic veterinary medicine, pathomorphology, and clinical traumatology. Based on the combination of macro- and microscopic signs, the mechanism of injury, the damage during lifetime, the features of thanatogenesis and the causal relationship between a gunshot wound and the onset of animal death were established.

Results and Discussion

Examination of the corpse of the first dog showed that it was a male (neutered) under 4 years of age, well-fed, body length 88 cm to the base of the tail. The body structure was proportionate and correct, the coat colour was pale brown, and there was an ear tag on the left ear. The hair cover was short and firmly attached to the hair follicles. The skin was grey and white. The coat was locally stained with blood behind the last rib, on the left side of the body (ventrally from the vertebrae), and on the right side of the body (lateral abdominal area) – behind the last rib, at the level of ½ of its length. One gunshot wound with a diameter of approximately 0.4 cm was observed in the skin of the above-mentioned areas, on the left and right sides of the body (Fig. 1).

A B

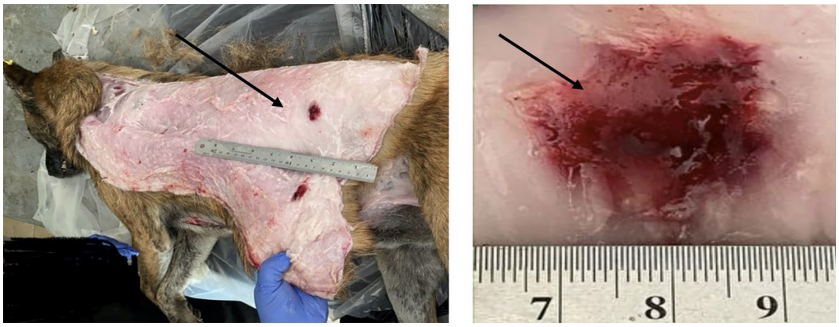
Figure 1. Entrance gunshot wound on the left side of the dog's body
***Note**: A – the black arrow shows the entrance hole from a gunshot wound; B – the size of the wound*
***Source**: photo taken by the authors*

The cornea, sclera, and conjunctiva of the eyes were light red in colour. The oral fissure was closed, vomit masses of white colour of doughy consistency were observed in the lumen of the oral cavity. The skin around the mouth slit was contaminated with vomit. The mucous membrane of the gums and oral cavity was moderately moistened, without damage, with natural pigmentation. The teeth were firmly held in their sockets. In the lumen of the nasal cavity, separate fragments of doughy masses (identical to the contents of the oral cavity), the mucous membrane of diffuse, light red colour was observed. The anal opening was clean, without pathological changes and damage. The integrity of bones and joints was not compromised. Algor mortis was clearly evident, with the lower jaw locked in place. There were no cadaveric spots or signs of decomposition of the corpse.

The results of a pathoanatomical autopsy of the first dog showed that during his lifetime he received a through gunshot wound with the establishment of a direct wound channel. Entrance gunshot wound on the left side of the body – area of the ventral edge of the first lumbar vertebrae; the wound channel ran from the cranial edge of the spleen dorsoventrally, in the wall of the duodenum (loops located at the level of the caudal edge of the pancreas); in the connective tissue of the cranial mesenteric lymphatic centre, in the wall of the jejunum; the exit gunshot wound was located on the right side of the body, at the level of ½ the length of the last rib.

C.S. Bartlett (2013), H. Capak *et al.* (2016) and B.B. Putra *et al.* (2017) based on morphological and functional changes in gunshot wounds, identified three zones within the wound channel: primary wound channel, contusion, and concussion. According to current research, the primary wound channel was caused by crushing, separation, and fragmentation of tissues along the axis the projectile trajectory. The diameter and contour of the same channel varied throughout its entire length. This is conditioned by the behaviour of the projectile and the anatomical characteristics of damaged tissues. Sometimes, with gunshot wounds, the resulting tissue defect is filled with wound detritus, crushed tissues, and blood. The contusion zone, or the zone of direct traumatic, primary necrosis, occurs when a projectile comes into contact with living tissues that are located in the immediate vicinity of the wound canal. As a result of the physical impact of the projectile on the tissues, they are the first to undergo necrosis at the time of injury or immediately, in the next few hours after it. A concussion zone is a zone of lateral impact that is directly adjacent to tissues that have completely lost their viability at the time of injury or in the next few hours after it. This was discovered during the resection of the dog's skin: on the left side of the body behind the last rib, in its dorsal part (the area of the ventral edge of the first lumbar vertebrae), the subcutaneous base and muscles (the psoas major muscle) around the wound channel were damaged and saturated with blood (diameter up to 2.5 cm) (Fig. 2).

On the right side of the dog's body, at the level of ½ the length of the last rib, the subcutaneous base and muscle tissue (oblique abdominal muscle) around the wound channel were saturated with blood. The above-mentioned injuries of the domestic dog were life-threatening injuries, which at the time of inflicting caused a threat to the animal's life. The injury caused pain that activated the gag reflex, which led to the ingress of vomit into the respiratory tract and their blockage. As the autopsy showed, the oesophagus and trachea contained vomit masses resembling a pasty mass (milky in colour with a light green tint) (Fig. 3). The mucous membrane of the oesophagus has acquired a greyish pink colour, the trachea – light red.



A B

Figure 2. General view of the entrance gunshot wound after resection of the skin on the left side of the dog's body

***Note**: A – the arrow shows the area of skin resection around the wound site; B – the area of soft tissue damage and blood impregnation*

***Source**: photo taken by the authors*

A B

Figure 3. Aspiration of the respiratory tract with vomit

***Note**: A – the arrows show the oesophageal mucosa with vomit of a domestic dog; B – the arrows show the presence of vomit in the lumen of the trachea, large and medium bronchi*

***Source**: photo taken by the authors*

The lumen of the trachea, large and medium bronchi was up to 80% clogged with pasty greyish white vomit with a light green tint. Light uneven colour, from light pink to dark red, darker red clearly defined areas that sunk above the general surface. Areas without clear contours of various shades of red were observed. Up to 30% of small bronchi contained vomit. Parenchyma had no content.

O.F. Pirgo (2024) pointed out that very often with gunshot wounds, animals experienced a state of shock. There are several mechanisms in its pathogenesis. This condition cannot be considered only hypovolemic. An important role is played by a pronounced pain syndrome and significant tissue damage, in which tissue mediators are released. Research by I.S. Zozulia *et al*. (2021) demonstrated

that central and peripheral pain receptors are involved in the perception of pain. Visceral pain occurs when the receptors located in the peritoneum are irritated and respond to damage. It does not have a clear localisation, and is often combined with signs of irritation of the autonomic nervous system: nausea, pallor of the skin, vomiting, palpitations, and sweating. At first, vomiting has a reflex character (due to irritation of the peritoneum), and then shock, hypoxia and intoxication of the body develop, the frequency of vomiting increases and becomes of central origin.

During the examination of the heart, no blood was observed in the atria, there was a moderate expansion of the lumen of the ventricles of the heart (right more) with a wall ratio of 1:4. No pathological changes were detected in the membranes of the heart. The blood was semi-liquid, dark red. The abdominal cavity contained liquid blood of dark red colour with admixtures of clots in a volume of up to 0.5 litres. On the surface of the serous membranes – blood clots. The visceral peritoneum and omentum contained moderate amounts of adipose tissue. Blood vessels had lower than average blood filling. Serous membrane of the abdominal wall, stomach, and intestines had a diffuse light red colour. In the abdominal wall on the left side of the body, an entrance gunshot wound with a diameter of up to 0.5 cm, oval in shape, was observed (Fig. 4).

A B

Figure 4. General view of the entrance gunshot wound from the serous membrane of the abdominal wall of a domestic dog

***Note**: A – the arrows indicate changes on the side of the serous membrane in the area of the entrance hole; B – the place of the entrance hole in the area of the abdominal wall on the left side of the animal*

***Source**: photo taken by the authors*

The wound channel passed through the dorsoventral surface of the spleen along the cranial edge of the organ, continuing into the wall of the small intestine loops and the connective tissue of the cranial mesenteric lymphatic centre. The exit gunshot wound was located in the abdominal wall on the right side of the body, oval in shape with a specific tissue deformity (Fig. 5). On the right side of the dog's body, at the level of ½ the length of the last rib, the subcutaneous base and muscle tissue (oblique abdominal muscle) around the wound channel were saturated with blood.

The stomach was then examined and found to be of normal shape, with no obstruction of the cardia or pyloric regions. The organ was half-empty, the mucous membrane of the bottom of the stomach was covered with contents identical to vomit found in the respiratory tract. Pronounced folding and light red colouration of the gastric mucosa were observed.

A B

Figure 5. General view of the initial gunshot wound after skin resection on the right side of the body of domestic dog

***Note**: A – the arrows in the figures indicate the location of the exit hole of the gunshot wound; B – the specific deformation of tissues in the area of the exit hole*

***Source**: photo taken by the authors*

The intestine (small part) was empty, the serous membrane had a light red colour, with the exception of two areas of rich dark red colour (at the level of the caudal edge of the pancreas), where through holes pass, which were a continuation of the wound channel. Damage to the integrity of the wall of the jejunum was established, the loop of which was located on the right side of the body. The edges of the holes were soaked in blood. Large intestine had no damage, light red colour.

The spleen remained unchanged in volume, diffuse light red in colour, with a wound channel running dorsoventrally from its cranial edge, which had stellate tears (more pronounced on the dorsal side), the edges of the tears were concave in the dorsoventral direction and saturated with blood. The liver was not enlarged in volume, with sharp edges, the organ had a diffuse light brown colour, the parenchyma was moderately moistened, the vessels were below average blood filling, and the parenchyma was distinctly granular. The gallbladder had a medium filling, bile of greenish mustard colour. The mucous membrane of the gallbladder was velvety, yellow in colour. The pancreas had a diffuse grey-pink colour, the vessels had below average blood filling. In the kidneys, the border between the cortical and medulla was well expressed, the colour of the cortical substance was light brown, the cerebral substance was greyish white. Bladder was pear-shaped, hollow, serous membrane of light red colour, mucous membrane of pale pink colour.

Pathoanatomical examination showed that the bones of the skull were dense and immobile, the mobility of the spine was preserved, and the spinal column had a natural configuration without signs of deformities or mechanical damage. In general, maintaining bone integrity in gunshot wounds in dogs was considered a prognostically favourable factor associated with a higher chance of survival with timely veterinary care, as reported by R.J. Fullington & K.M. Otto (2017). However, the results of research by J.R. Lewis *et al*. (2008) and M. Grela *et al*. (2021) indicate that penetrating damage to internal organs or involvement of anatomically critical areas significantly impairs the prognosis regardless of bone condition, which was confirmed by the results obtained in the current study.

During histological examination of the entrance gunshot wound, it was found that the structure of the affected tissues was dominated by pronounced necrodystrophic changes and

significant haemodynamic disorders. In the parenchymal-stromal elements of soft tissues, an intense layering of powder combustion products was visualised: soot and small individual dust particles. Localisation of these elements had a specific character: the surface was characterised by the fact that the bulk of soot and microparticles of gunpowder ended up in the epidermis and surface layers of the dermis, and with deep localisation, due to the mechanical action of the shot, there was a delamination of the surface layers of the skin, which ensured the penetration of foreign particles into the dermis. They were infiltrative and concentrated perifollicularly (around the hair follicles). In addition, micro particles of gunpowder were found in the sebaceous glands. Morphological changes were recorded in the reticular layer of the dermis, which were manifested by pronounced oedema and destructive changes in the vascular bed. Uneven connective tissue oedema was noted. Disorganisation of collagen fibres was observed: their thick bundles were focally detached and lost contact with subcutaneous adipose tissue. The compression of fibres was also observed, leading to histoarchitectonic disruption. In the affected areas, the walls of blood vessels were homogenised, the endothelium was desquamated, and blood clots were present in small vessels.

When examining muscle tissue in the wound area, a combination of alterative changes, haemodynamic disorders, and mechanical stratification of muscle fibres was recorded. Diffuse and focal haemorrhages were also detected. In the areas of haemorrhagic infiltration, the structure of muscle fibres was sharply disrupted: there was a loss of sarcolema, infiltration of sarcoplasma by red blood cells, and the absence of nuclei. In relatively preserved fibres, the absence of transverse striation was noted, which is evidence of deep destructive changes in myofibrils. The established morphological features were sufficient to determine the mechanism of wound formation and its differentiation from other types of injuries.

Based on the conducted pathomorphological study of Case No. 1 (through gunshot wound of the abdominal organs of a domestic dog), the following key aspects were established: morphological parameters of the entrance and exit holes, and the nature of the wound channel, most likely indicate the use of small-calibre firearms using pellets with a diameter of approximately 0.5 cm. A direct causal relationship was also established between the gunshot wound caused and the death of the animal. The specified wound was classified as an injury that caused acute, life-threatening pathological phenomena at the time of infliction. The death of the animal occurred as a result of the combined action of traumatic factors. The leading pathological processes were: acute internal bleeding (haemoperitoneum) caused by multiple violations of the integrity of the parenchymal and hollow organs of the abdominal cavity (spleen, duodenum, jejunum) and obstructive asphyxia (mechanical obstruction of the airways by aspirated vomit), which led to obstructive atelectasis of the lungs. The detected signs (hyperaemia of the cornea and sclera, anaemia of internal organs, the presence of vomit in the respiratory tract) confirm that the injury was in vivo, accompanied by intense pain and activation of protective reflexes (vomiting) shortly before death. This case illustrates the need for a detailed sectional study to identify all links in thanatogenesis, which is key to an objective forensic veterinary assessment.

The results of previous research and studies by other scientists indicate the need for pathomorphological research to consider such signs as: damage to organs responsible for vital functions of the body, the area and severity of damage to various organs and tissues; the degree of blood filling of vessels of parenchymal

organs; the state of blood in the vessels. The development of a blood clot in the area of vessel damage (local haemostasis) helps to stop bleeding. However, during acute massive blood loss, damage to large-calibre vessels and traumatic injuries to a large volume of tissues, excessive blood hypercoagulation becomes an independent pathogenic factor and can lead to the development of disseminated intravascular coagulation (DIC) (Skrypka *et al.*, 2020; Yatsenko, 2023).

In the second case, a study was conducted on a domestic dog (female), 5 years old, mixed breed, average body condition. The body structure was proportionally correct; the coat was black and grey with a white colour of the ventral neck and underbelly (grizzle). Hairline was medium length, firmly attached to the hair follicles. The skin was grey and white. The integrity of the bones and joints was not compromised. There was no discharge from natural orifices. The anus was clean, without pathological changes and damage. The conjunctiva of the right eye was red with haemorrhages. The integrity of the membranes of the right eyeball within the anterior pole was damaged with a hole and a dark red (almost black) border along the edge, with blood in the form of a thin crust on the surface (Fig. 6A). The conjunctiva of the left eyeball was unevenly dull red in colour, with no damage to its integrity. The lumen of the nasal cavity was free, the mucous membrane was diffuse light red in colour (Fig. 6B). The oral cavity was closed.

A B

Figure 6. External pathomorphological changes in the head area of a domestic dog after a bullet wound

***Note**: A – the arrows in the figures indicate haemorrhagic impregnation of the right eyeball; B – hyperaemia of the nasal mucosa*

***Source**: photo taken by the authors*

The mucous membrane of the gums and oral cavity was moderately moistened, without damage and layers with the existing natural pigmentation. The root of the tongue and the pharyngeal mucosa were light red with haemorrhages. The teeth were firmly held in their sockets with moderate enamel wear and pronounced tartar build-up.

Algor mortis and rigour mortis were well expressed. However, there were no cadaveric spots or signs of decomposition of the corpse. During skin resection in the head area, dark red discolouration was observed in the subcutaneous tissue and muscles around the right eyeball, root area, and bridge of the nose. The periorbital, intra- and extraorbital fat bodies have become gelatinous in consistency, saturated with bloody fluid. The muscles under the eye (circular eye muscle) had a rich dark red colour, high humidity in the form of a triangle (Fig. 7).

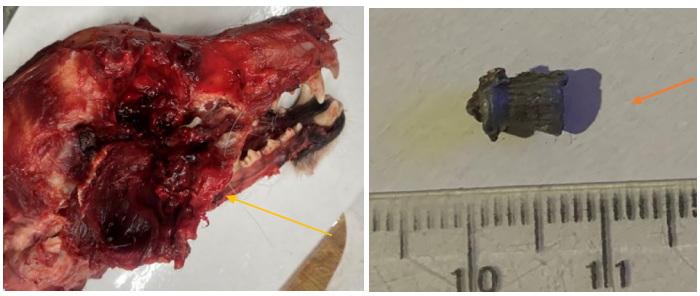
A B

Figure 7. Pathomorphological changes during skin resection in the head area of a domestic dog after a bullet wound

***Note**: A – the arrows indicate haemorrhagic impregnation of periorbites, intra- and extraperiorbital fat bodies; B –haemorrhagic impregnation and the entrance wound opening of the right eyeball*

***Source**: photo taken by the authors*

During the examination of the animal's masticatory muscles, diffuse haemorrhage with the formation of dark blood clots was observed (Fig. 8A). The same diffuse haemorrhage was found in the pterygoid muscle. A metal object resembling a shotgun pellet, up to 1 cm long, with signs of moderate deformation, was found and removed from the thickness of the latter (Fig. 8B).



A B

Figure 8. Pathomorphological changes in the masticatory muscle area caused by a foreign object

***Note**: A – the arrows indicate haemorrhages in the masticatory muscles; B – a metal object removed from the masticatory muscle tissue*

***Source**: photo taken by the authors*

The lungs were diffuse light red in colour, with vessels of moderate blood filling. Lumen of the trachea, bronchi and alveoli had no contents. When examining the heart, the expansion of the lumen of the right atrium (maximum) and ventricle was observed as a result of their blood filling, postmortem blood clotting was pronounced. No pathological changes were detected in the membranes.

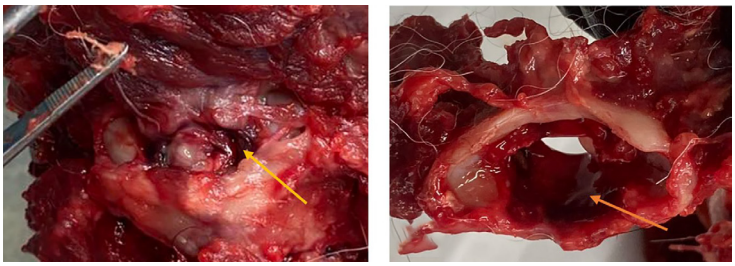
Abdominal cavity had no foreign contents. The placement of organs was anatomically correct. The serous membrane of the digestive tract and the greater and lesser omentum were diffusely light red in colour. The visceral peritoneum and omentum contained moderate amounts of adipose tissue. Blood vessels had moderate blood filling. Serous membranes were moderately moist and smooth. The stomach had a

regular shape. The patency of the cardiac and pyloric parts was not disturbed, the organ was below average filling, contained dirty-grey fluid, the gastric mucosa was light red, and greyish pink in other areas. The intestines were empty, the serous membrane was light red, the mucous membrane was pale pink, covered with a moderate amount of translucent mucus. The spleen was not enlarged in volume, pink with a brownish tinge, the edges were pointed, there was no scraping of the parenchyma, blood vessels of medium filling. The liver was not enlarged in volume, brownish red, the parenchyma was moderately moistened, the vessels are of medium blood filling, the parenchyma was distinctly granular, the edges were rounded. The gallbladder had a medium filling, bile had greenish mustard colour. The mucous membrane of the gallbladder was velvety, yellow in colour. The pancreas was characterised by a diffuse light red colour, and the vessels had below-average blood filling.

In the kidneys, the border between the cortical and medulla was not pronounced, the colour of the parenchyma was red brown. The bladder was pear-shaped, empty, the wall was thickened, the serous membrane was light red, the mucous membrane was uneven dull red, and barely noticeable haemorrhages were noted. The lymph nodes of the cranial mesenteric lymph centre were not enlarged, pink, and the parenchyma was moderately moist. The wall of the uterus, uterine horns and ovaries were diffusely red in colour. The animal was pregnant (up to 2 weeks of gestation, 8 foetuses were counted).

The bones of the skull were dense, connected to each other motionless. The periosteum was uneven from light pink to dark red. The membranes of the brain were uneven dark red in colour, in the area of the basal surface of the brain-blood clots, brain tissue of gelatinous consistency, vessels filled with blood. A focal subdural haematoma was found in the area of the visual intersection and the appearance of a diffuse subdural haematoma of the brain in the caudal direction, which went beyond the brain cavity, reaching the subdural space of the spinal canal (1-2 cervical vertebrae).

Spinal mobility remained within the normal range. Vertebral column had no deformations and damage, natural configuration. The integrity of the spinal cord was not compromised. The spinal canal of the atlas contained a blood clot, and the meninges of this area had an uneven red colour (imbibition) (Fig. 9).



A B

Figure 9. Changes in the spinal canal area of the atlas

***Note**: A – the arrows indicate the spinal canal in the atlas region containing blood clots; B – blood impregnation of the medial (inner) wall of the atlas*

***Source**: photo taken by the authors*

The pathomorphological study of the Case No. 2 of a blind gunshot wound to a domestic dog provided the following conclusions: the death of the animal occurred as a result of the

development of critical pathological processes threatening the body, namely: severe traumatic brain injury, which led to the formation of massive intracranial and spinal haematomas and secondary brain oedema. A direct causal relationship has been established between mechanical damage caused by the action of pellet weapons and the onset of animal death. Detected injuries (violation of the integrity of the eyeball, subdural haematomas of the brain and spinal cord, brain oedema) were classified as severe injuries. This case was an illustration of the application of objective morphological criteria to confirm the fact of animal cruelty. The degree of damage to the central nervous system indicates the presence of intense pain and irreversible changes, which is a sufficient basis for appropriate legal qualification of actions.

Analysis of the results of studies of two cases of gunshot wounds of animals showed that when classifying trauma as severe or fatal, massive blood loss or the development of disseminated intravascular coagulation syndrome (DIC) is not always the leading pathogenetic mechanism. The results obtained are consistent with the data by A.M. Perebetiuk & V.V. Biktimirov (2003), who noted that when fired at close range from a gas barrel weapon, blood vessels are the first to suffer. Changes in them depend on the area of attachment of vessels to the wound, massive thrombosis is noted in large blood vessels, and due to plasmorrhagia, the walls of these vessels are homogenised. C.S. Bartlett (2013) found that in more remote areas from the wound, in the vessels of the microcirculatory bed, there is a violation of the rheological properties of blood in the form of stasis and microthromb development. The permeability of the walls of blood vessels becomes high, which is confirmed by diapedetic haemorrhages. Changes that occur in the wound area when fired at point-blank range from a gas barrel weapon are also characteristic of injuries from a firearm.

M. Risselada (2017) emphasised that penetrating traumatic injuries in dogs, in particular gunshot wounds, can be accompanied by minimally pronounced external morphological changes in the presence of significant internal injuries, which complicates their initial visual assessment. L. Miller *et al*. (2018) showed that in service dogs that suffered combat and gunshot injuries, deaths were most often caused by damage to internal organs and the development of critical pathological conditions, and not by the nature of external wounds. From a forensic veterinary standpoint, this is of fundamental importance, since an objective assessment of the severity of gunshot damage is possible only based on the results of a full-fledged sectional study with an analysis of the wound canal and damage to internal organs. The results obtained in this paper confirmed the need for a comprehensive pathomorphological approach to establish the mechanism of injury, thanatogenesis, and causal relationship between damage and the onset of animal death.

Thus, the results of pathomorphological and histological examination of two cases of gunshot wounds in dogs demonstrated significant variability in the localisation of injuries, mechanisms of traumatic action, and morphological manifestations of tissue and organ damage. In both cases, the characteristic signs of gunshot trauma with the formation of wound channels, pronounced alterative and destructive changes in soft tissues, vascular disorders, and secondary pathological processes that were crucial in the development of fatal consequences were established. The obtained morphological data helped to objectively assess the severity of injuries, their lifetime nature, and the relationship between the localisation of trauma and the development of critical pathological conditions. The presented results expanded the available information on the pathomorphological features of gunshot wounds in dogs and can be used in veterinary

pathoanatomy, forensic veterinary practice, and in the preparation of expert opinions.

Conclusions

The study of two cases of death of domestic dogs due to gunshot wounds allowed systematising the key pathomorphological criteria necessary for an objective forensic veterinary assessment of animal cruelty. In both cases, the presence of multiple serious injuries caused by firearms was unquestioningly qualified as ill-treatment that led to the death of animals. Despite the different locations of the primary injury (abdominal cavity in Case No. 1; eyeball and central nervous system in Case No. 2), critical, life-threatening pathological processes developed in both situations. In particular, Case No. 1 illustrated the rapid development of haemorrhagic shock due to internal bleeding. Case No. 2 recorded neurological complications – fatal consequences of traumatic brain injury (haematomas, brain oedema), which caused irreversible changes in the central nervous system. The complex of detected histomorphological changes (pronounced necrodystrophic processes, haemodynamic disorders, and destruction of muscle fibres) indicated the presence of a gunshot wound. The characteristic localisation of gunpowder combustion products in the deep layers of the dermis and specific changes in the vascular bed were pathognomonic signs of the effects of concomitant factors of a firearm shot from close range (contact or near-contact shot). The identified criteria allowed differentiating this wound from injuries of other origin.

Based on a detailed pathoanatomical study in both cases, a direct causal relationship was clearly established between the action of the traumatic factor (shot) and the onset of death, which is a key criterion for qualifying a crime. Morphological features, such as ante-mortem tissue reactions, hyperaemia of the mucous membranes, organ anaemia, and obstruction of the respiratory tract by vomit, confirmed that the animals experienced intense pain and significant suffering shortly before death. The described cases emphasised the need for a comprehensive approach (including pathoanatomical and histological examination) to fix all injuries and determine the exact mechanism of death, which ensures the scientific validity of forensic veterinary examination. They demonstrated that a comprehensive approach is needed to objectively establish the truth in the forensic veterinary examination of gunshot wounds. A detailed study of all body systems allows not only stating the fact of injury, but also accurately establishing the pathophysiological chain of events that led to death, which is of key importance for forensic qualification of animal cruelty.

The prospects for further research are to expand the sample of forensic veterinary cases of gunshot wounds in domestic animals to clarify the spectrum of morphological variants of injuries and features of thanatogenesis, depending on the type of weapon, the distance of the shot, and the location of the injury. It is advisable to use in-depth histochemical, immunohistochemical, and molecular methods for objectifying the lifetime of injuries and temporal assessment of injuries, which will increase the evidentiary value of expert opinions in criminal proceedings related to animal cruelty.

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Conflict of Interest

None.

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Патоморфологічне дослідження трупів собак, які загинули внаслідок вогнепальних поранень

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Анотація. Актуальність дослідження зумовлена збільшенням частоти застосування вогнепальної зброї з ураженням тварин під час воєнних конфліктів. Як показує практика, більшість вогнепальних поранень – це випадки жорстокого поводження з тваринами. Саме тому, судово-ветеринарний експерт повинен правильно оцінити не лише вогнепальне поранення, але й обставини, пов'язані зі стрільбою. Метою цього дослідження було виявлення та з'ясування патоморфологічних змін, які виникають в органах і тканинах собак у зоні вогнепальної рани, при пострілі зі ствольової зброї. В рамках проведення судово-ветеринарної експертизи було здійснено патологоанатомічний розтин двох безпритульних собак, що загинули від вогнепальних поранень. Перший собака свійський за життя отримав наскрізне поранення органів черевної порожнини, причиною загибелі другого собаки стало сліпе поранення в голову. З'ясовано, що у тканинах вогнепальної рани переважали дистрофічні та некротичні зміни, а також розлади гемодинаміки. В скелетній м'язовій тканині реєстрували вогнищеві та дифузні крововиливи, які локалізувались між розшарованими м'язовими волокнами. Поряд із цим, відмічали вогнища геморагічної інфільтрації. В просвітах судин великого калібру виявляли масивне тромбоутворення, але основні зміни відмічали в мікроциркуляторному руслі. Патологічні зміни, які фіксували на тілах загиблих собак, відносилися до важких тілесних ушкоджень. Вони призводять до загрозливих для організму патологічних процесів і закінчуються загибеллю тварини. Отримані результати дають можливість поглибити знання судово-ветеринарних експертів із питань ураження тварин вогнепальною зброєю, а виявлені зміни можуть слугувати для диференціювання ран, спричинених дією такої зброї, від ран іншого генезу

Ключові слова: судово-ветеринарна експертиза; вхідне поранення; вихідне поранення; вогнепальна зброя; розтин

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