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# The quality of life after a major surgical intervention: mandibulectomy

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## Abstract

*The tumours localised on the mandibular bone are one of the pathologies with a serious prognosis, difficult to approach for the veterinary clinicians and to accept by the owners. The treatment of choice for most of the cases is the surgical one. The surgical technique varies depending on the portion that has to be removed. For the clinicians, this intervention represents a challenge requiring solid knowledge of anatomy and possession of a numerous and diverse instruments. For the owners, the decision to choose the surgical solution is difficult to make, the main threats being the change of the animal's facial aspect after the intervention and the fear that their quality of life will be affected. However, the results are the most time satisfactorily: the patients not only live for many years after, but they get used very fast to make the prevention of food and to swallow, living a normal life. The only aspect that cannot be controlled is the risk of a recurrence.*

**Key words:** mandibulectomy, oral cavity, tumours,

## Introduction

Tumours of the oral cavity are the fourth most common type of tumor in dogs and cats, accounting for 5.4% of all malignant neoplasms. (1) The gingival mucosa is most commonly affected, but the oral, mucous membrane, soft palate, tough palate and tongue can also be affected. (2) Major surgery is the main indication for oral and maxillofacial malignancies and is surprisingly well tolerated by dogs and cats. The quality of animal life after mandibular surgery is excellent.

### Material and method

Tumours of the oral cavity, either malignant or benign, sometimes involving the mandible and the periosteum, require excision of the affected bone segment by mandibular surgery. The bone fragment which has to be resected is determined by the localisation of the tumor process. Tumours located unilaterally or bilaterally in the rostral part of the mandible can be excised without difficulty. Partial mandibulectomy is also indicated in the treatment of certain mandibular fractures and in mandibular osteomyelitis. Mandibulectomy is the main surgical procedure indicated to remove fragments that cause pain in fractures whose primary ossification has failed. (5) Depending on the excised segment, mandibulectomies are divided into: partial (unilateral or bilateral), rostrale, or including a portion of the mandible body, total (unilateral resection of one of the two mandibular bones) or, most radically, the total resection of one mandible and a portion of the other. (3,4)

The eight-years-old labrador dog was presented at the Surgery Department of the Faculty of Veterinary Medicine in Iași after an aggressive recurrence of the oral tumor. This tumor was previously excised and relapsed faster than before in less than two months.

A radiological and anatomopathological examination was required for an accurate diagnostic. The radiological examination (Fig. 1) confirmed the presence of a tumour mass that affected the mandible bone, and the anatomopathologic one provided the diagnosis of gingival fibrosarcoma. In order to perform the surgery, the dog was placed in lateral decubitus on the opposite side of the tumor. (Fig. 2)

For a better exposure of the region, the mouth was opened with a mouth speculum. After trimming the hair in the mandibular region, the asepsis was secured with an iodine solution. Anesthesia was a combination of xylazine-ketamine, premedicated with atropine. The first operative time was consisted in the isolation through a firm incision in an elongated flap of the

labial lobe and of the right sublingual recess, using an electric scalpel, keeping strictly the trace and integrity of the Wharton duct and the monostomal sublingual gland (Fig. 3). The take-off of the soft structures (muscles) from the lateral and medial sides of the segment that was subsequently dissected was then made. Hemostasis was provided by ligation of the main vessels, but an essential role in stopping the bleeding due to small and medium sized vessels was the use of the electrocautery and the bipolar twin.

The mandibular bone segment and the soft tissues covering it were both removed by (using) a pneumatic oscillating blade osteotomy. The mandibula was resected from mandibular symphysis level (Fig. 4) to the aboral edge of the mandibular body. For better viewing during surgery, it was necessary to use a surgical vacuum cleaner. After removal of both, the mandible bone segment and the tumour, the suture of the lining of the mucosa and the sublingual mucosa was made using a PGA 2/0 resorbable suture thread in separate points. (Fig.7)

Immediately after surgery, tumour mass fragments were harvested and fixed for 72h in 10% formaldehyde solution. After fixation, tissue fragments were embedded in paraffin, cut at 5 micrometers, stained by the Masson trichromic method and examined with a Leica DM 750 optical microscope, the images being taken up with a Leica Application Suit (LAS) digital software version 4.2. (Fig. 8).

## Results and discussions

After the surgical procedure, the dog received prophylactic antibiotic therapy with a broad spectrum antibiotic from the penicillin group for 3 days and analgesics, both parenterally. The postoperative diet, starting on the day after surgery, was consisted of liquid food for two weeks. Some of the most common postoperative complications are wound dehiscence, in which case it is recommended to perform secondary sutures, postoperative infections and tumour recurrence in the case of micrometastases.

Histologically, at the surface of the tumour, diffuse acanthosis, characterized by proliferation and hypertrophy of the spinous cells were observed. Thick cells are large, well-contoured and with highlighted desmosomes. No superficial keratinized layer was observed. Tumor tissue was organized into compact (massive) area, consisting of spindle cells with large, irregular, vesicular nuclei, powdered chromatin and a prominent nucleus. Numerous mitotic figures and syncytia have been observed. Connective fibers have a chaotic orientation, either in the form of cords or concentric. Neoformation vessels are present, and the stromal tumor is poorly developed. As a cellular type and architecture of the tumor, the histopathological exam revealed fibrosarcoma, associated with an acanthosis of the oral mucosa. (Fig. 9)



**Fig. 1.** Radiological examination of the head region - the tumor process invaded the bone tissue

**Fig. 2.** The macroscopic aspect of the tumour



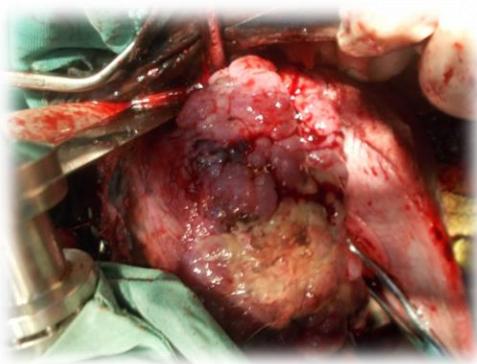
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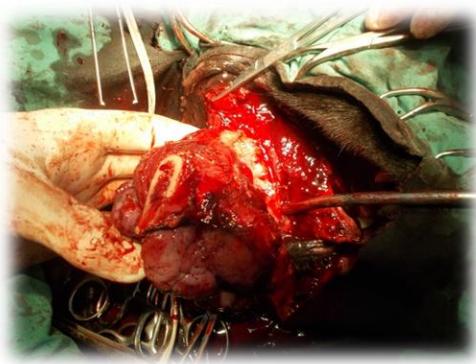
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**Fig. 3.** The incision of the labial mucosa

**Fig. 4.** The osteotomy of the mandible at the symphysis region



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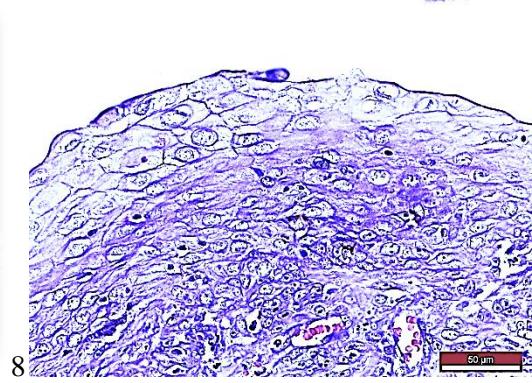
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**Fig. 5.** Osteotomy of the mandible at the condyle region

**Fig. 6.** Hemostasis achieved by coagulating blood vessels and aspiration of fluids



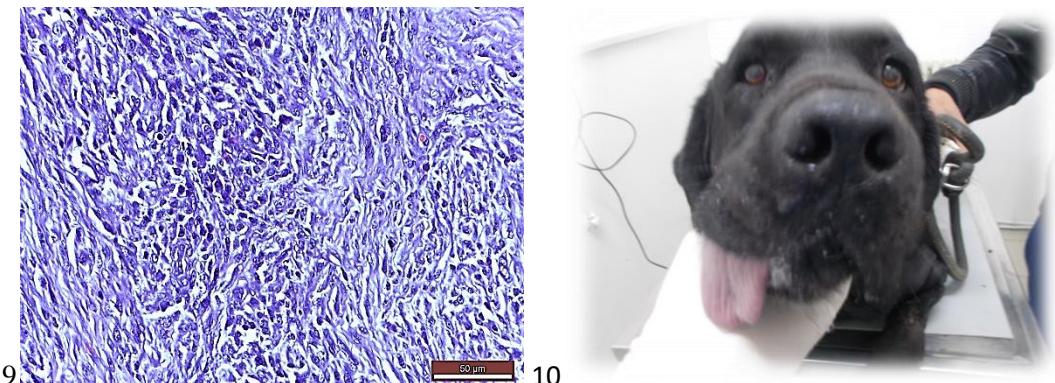
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**Fig. 7.** The suture made in separate points using 2/0 resorbable thread

**Fig. 8.** Achantosis. Oral Mucosa. Tricromic Masson Stain



**Fig. 9.** Fibrosarcoma. Tricromic Masson Stain

**Fig. 10.** Overall appearance two weeks postoperatively

### Conclusions

The general aspect of the animal is determined by the dimensions of the excised portion. If hemimandibulectomy is made to the level of the canine, the differences are almost insensible. If the excised portion exceeds the lower canine, the tongue tends to stay out of the oral cavity. (Figure 10)

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# **Microbiological quality of chicken meat: *Campylobacter* relevant public health problem**

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## **Abstract**

*Campylobacter* is undoubtedly an important public health problem that European legislation (Directive 2003/99 / EEC) establishes as zoonotic agent to be monitored, with mandatory notification of the number of isolates, registration of cases, and the provision of information to Member States. However, despite its high prevalence, so far they have not established measures for its reduction or the obligation to implement control plans. Unlike *Salmonella*, which, most likely, thanks to community prevalence reduction targets and national control plans, still, for years, a marked downward trend in the number of cases in the EU. Consumption of contaminated chicken meat, mainly by *Campylobacter jejuni* is the leading cause of human campylobacteriosis in Europe, so it EFSA considered necessary to establish measures to reduce *Campylobacter* in chicken flocks. According to the European Food Safety Agency (EFSA), a real incidence of 9 million annual cases of campylobacteriosis in the EU is estimated. The average prevalence of *Campylobacter* in broiler flocks at Community level is 71.2%, with 75% in the case of Romania, it is therefore a major problem. A lot of birds that come to slaughter excrete the bacteria in their feces and is very difficult to prevent, during processing, contamination of carcasses and abattoir equipment occurs. Therefore it is important to reduce the bacterial load in birds before arrival at the slaughterhouse. As the reports of the EFSA and the European Center for Disease Prevention and Control (ECDC), based on the results of monitoring activities of zoonoses (diseases transmitted naturally from vertebrate animals to man diseases), carried out in 2015 in 32 European countries (28 Member States and four non-Member States), campylobacteriosis is the most commonly reported zoonosis. In view of this trend, the European Union is preparing a draft regulation that will come to amend the current legislation for the control of *Campylobacter*. Those rules shall establish a process hygiene criterion for *Campylobacter* in broiler channels to control possible contamination with this bacteria during the slaughter stage. This draft European regulation also establishes a contamination value above which corrective action is required to maintain hygiene, according to food legislation (20% of chicken carcasses with a level of *Campylobacter*  $\geq 1000$  cfu / g, for 2025). The European Commission considers that this approach should be implemented gradually and tighter over time.

**Keywords:** *Campylobacter*, public health, EFSA, chicken meat

## **Introduction**

Given the important role of the species in the genus *Campylobacter*, the World Health Organization has developed over time a partnership with various stakeholders, to develop general policies to promote food safety. These include specific recommendations and technical expertise throughout the entire food chain, from production up to (1.2).

The WHO reports sporadic episodes of recorded foodborne diseases, however, believes that their incidence is due to the fact that these micro-organisms and, thanks to its properties, the difficulty (7.8).

Specialty reports mentioning another important aspect which argues that it is almost impossible to determine with certainty all sources of contamination with microorganisms. Wide distribution of the species in the genus *Campylobacter*, it hinders the development of effective control strategies along the food chain. However, in countries that have adopted specific measures

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to reduce the prevalence of *Campylobacter* in poultry, it was observed a reduction in the number of episodes in humans food with Community rules (1, 3)

*Campylobacter* infections are the most common cause of bacterial gastroenteritei in industrialized countries. Annual incidence varies between countries and is growing in several of these (2,3).

## **Material and Methods**

Research has been carried out within the framework of a slaughter of birds in Romania. The samples were processed in the laboratory of Microbiology and Food Control Laboratory, Faculty of Veterinary Medicine of Iași.

Research was conducted during the period 2016-2017, and had as objectives:

-isolation of *Campylobacter spp.* strains from broiler cecocolic junction;

-identification of the isolated strains on the basis of cultural, biochemical;

-to establish the incidence of *Campylobacter* species isolation from birds and poultry carcasses.

Assessment of species *Campylobacter spp.* has been of programs ANSVSA:S.R. ISO 10272/2007.

During the period 2016-2017 have been sampled and analysed 25 samples of cecumuri from broiler, and 25 samples of carcasses of broilers.

At the present time are being offered more possibilities for isolation of *Campylobacter* species. Insulation standard distributed by 2015, OIE Manual recommends mCCDA environment but may also be used in other alternative means of containment. Corry et al. (2003) environments and uses selective groups for *Campylobacter* selective medium: with blood (Agar Preston Agar, Agar, Skirrow was Butzler, Campy-cefex) and selective medium with charcoal (mCCDA agar agar, Karmali, CAT) plus various antibiotics: cephalosporins (usually cefoperazonă), combination of Vancomycin and trimethoprim but also some antimicrotice such as cicloheximida or amfoteracina B. The purpose of these add-ons for antimicrobial is to inhibit as much of the microflora of contamination and confined the species *Campylobacter jejuni* and *Campylobacter coli*.

In the case of our study, for the bacteriological examination were required:

1. Materials: culture media and reagents: broth medium: liquid Bolton broth Brucella, solid media: Agar agar modified (mCCD coal cefoperazonă deoxicolat; with agar blood agar Karmali; Colombia;
2. Oxidase reagent strips for:-1-4-Tetramethyl-Phenylenediamine dihidroclorică
  - hippurate hydrolysis reagent for detecting-sodium hipurat solution;
  - ninhydrin solution 3.5%-mixture of ninhydrin, acetone and butanol;
  - Indoxyl acetate-blend of indoxyl acetate and acetone impregnated in filter paper discs.
3. Sterile Petri plates and glassware: glass and plastic bottles, test tubes and pipettes, Erlanmayer, Pasteur pipettes, chance of sowing.
4. Microbiology lab-specific equipment: automatic adjustable pipetor anaerostat Cystocell, optical microscopes, microscope ML4 phase-contrast (required to observe mobility characteristic bacteria of the genus *Campylobacter*).
5. Bag systems with gas generators

Sampling cecums from broiler chickens was done in under the 'program of surveillance, prevention and control of animal diseases, those transmissible between animals and humans, animal protection and environmental protection' established by ANSVSA according to the EU rules into national law.



**Fig.1.** Cecums bird collected, washed and antiseptizate

Samples were collected at random for each lot of animals culled, 25 were taken from 25 cecumuri, broiler, chosen on the cutting line while abiding by the rules on traceability. Cecums taken must be intact, neînțepate and filled with content. Cecums are subject to antisepsiei with alcohol followed by drying (Fig. 1.).

After cutting the apex, the intestinal content cecal junction is made using a Pasteur pipette, sterile. Carcasses from which the samples are taken from the cecumuri must not succeed in succession line cutting and sampling should be avoided in the first part of the sacrificed.

### Results and Discussions

*Campylobacter* there are major food safety perspective when they colonize the digestive tract to a flock of birds, becoming a carrier. The possibility of contamination of the meat after slaughtering the birds represent a risk to human health. Because of these risks, imposed biosecurity measures to prevent the introduction of species of *Campylobacter* in poultry or halls to avoid dissemination of these microorganisms in the environment or to other flocks.

Growth medium is always slow, the colonies being observed after a long incubation. agar Karmali, after a 48-hour incubation, bacterial culture has had a development.

Typical of *Campylobacter jejuni* colonies had a flattened appearance, with irregular edges, with shades of grey, sometimes with metallic luster and that invades as a surface film geloses. Smears made from typical colonies on selective, were stained by Gram's method. In the optical microscope examination, observed gram-negativ incubation, easy-looking bracket or a comma with dimensions of 1-2  $\mu\text{m}$ .

To identify the species of *Campylobacter jejuni* and *Campylobacter coli* differentiation has been made the test of hipuratului hydrolysis of Na as campilobacterii strains analyzed, who left positive test hipuratului Na hydrolysis have the species was *Campylobacter jejuni* were negative and the strains of *Campylobacter coli*.

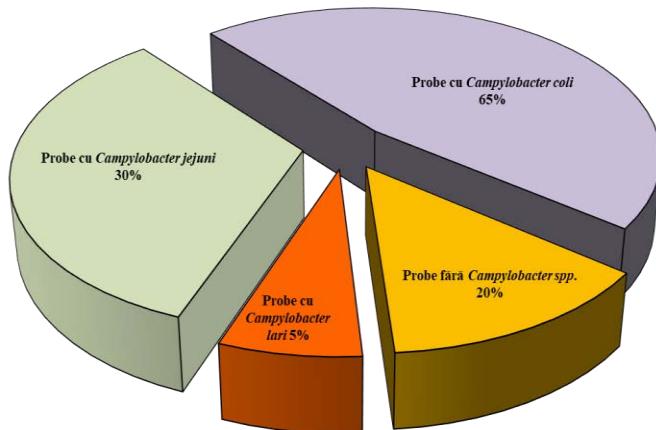
As a result of microbiological analyses have identified 23 (92%) of samples positive for *Campylobacter*. The intestinal content of positive cecums have been isolated from 40%) 10 (strains of *Campylobacter coli*, a number of 8 samples (32%) strains of *Campylobacter jejuni* and 5 samples (20%) strains of *Campylobacter lari* ( Table 1.).

**Table 1.** The presence of *Campylobacter* in broilers cecums

Investigate d samples	Positive samples Nr.	Isolated strains					
		<i>Campylobacter coli</i>		<i>Campylobacter jejuni</i>		<i>Campylobact er lari</i>	
%	Nr.	%	Nr.	%	Nr.	%	
25	25	2	9	1	4	8	5
	3	2	0	0	4	2	20

Of the 25 samples of carcasses collected from slaughterhouse and subjected to investigation in 20 samples detected *Campylobacter* spp. and 5 samples were free of campylobacterii.

*Campylobacter coli* was detected in 13 samples subjected to investigation in 6 samples detected *Campylobacter jejuni* in a single sample. detected *Campylobacter lari*.



**Fig.2.** The share occupied by *Campylobacter* spp. in the 25 samples analysed

Salmonellosis in recent years have been the main cause of food toxifications, statistically it has been complicated by the evolution of campylobacteriosis, particularly those of the genus *Campylobacter jejuni* and *Campylobacter coli*, responsible for more than 8 million cases annually in the EU to the people. Food Standards Agency (F.S.A.) of the United Kingdom ordered the imposition of control over trade in the commercial network with possible infected birds as well as the publication of the results of supervision, but along with campylobacteriozelor companies poultry supply agency (5.6).

Some studies show that a percentage of 4% to 13% of positive cases occur after packing, what allows to assume that there is a cross-contamination in both process and the finished product handling by workers. From the data collected could ascertain that infection season has a greater frequency in the summer months.

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The leading manufacturers of broiler tries to improve biosecurity measures during natural movements finished products processing or by vaccination. Research at the University of Liverpool have shown that vaccinating poultry can be done, just that the immune response does not develop fast enough, the slaughter at about 6 weeks. Another way to reduce bowel colonisation was the use of essential oils, probiotics or bacteriofagi.

So far there have been made targets of reducing his *Campylobacter*, unlike *Salmonella*, which thanks to the EU's objectives of reducing prevalence and national plans was a clear trend of decrease in the number of cases in the U. S (6, 7).

If it has them in consideration both sanitation solutions chemical ban after evisceration and that consumer marketing of frozen birds represent a rather low percentage in relation to fresh or chilled meat, it can be said that it will It remains a problem for the population campylobacteriosis.

### Conclusion

1. Microbiological investigations were conducted in accordance with the guidance provided in the 'program of surveillance, prevention and control of animal diseases, those transmissible between animals and humans, animal protection and environmental protection' established by ANSVSA and on the isolation and identification of *Campylobacter* in broilers.
2. The results of the cecums confirms that the birds are carrying campilobacterii constituting a significant source of contamination during evisceration the carcasses and heighten the risk of toxinfestations in humans food following handling and consumption of contaminated meat and insufficiently processed.
3. The largest share is taken by the *Campylobacter coli* samples analyzed, harvested both from cecums level and at the level of animal carcasses.
4. In view of this trend, the European Union shall prepare a draft regulation to amend the legislation will come into force for control of *Campylobacter*.
5. This regulation establishes the european project also worth over contamination requiring corrective action to maintain hygiene, in accordance with the law of food (20% of chicken carcasses with *Campylobacter*  $\geq 1000$  cfu/g, for 2025).

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## **Protocol of polyovulation to donor buffaloes (Romanian Buffaloes)**

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### **Abstract**

*Buffalo has a significant role in the agricultural economy of many developing countries by providing milk, meat and draught power. The breeding season for Romanian Buffaloes in the major buffalo rearing countries appear to extend from September to March. Compared to cattle, buffaloes exhibit delayed puberty in both males and females, poor estrus expression in a greater proportion of females. The main problem affecting the productivity of buffaloes is the long calving interval due to delayed postpartum estrus and delayed puberty. This is due to the difficulty in detecting estrus in this species on account of poor signs of estrus, such as silent heat, quiet ovulation or subestrus. Polyovulation is an important step in embryo transfer biotechnology. Over the years, techniques associated with embryo transfer have had many uses, especially in research. The widespread use of this technology in animal breeding schemes, however, is relatively recent. Successful embryo transfer requires manipulation of oestrus and ovulation of the donor and synchronization of these events with those of the recipients. A brief review of the physiological and endocrinological aspects of the oestrous cycle will contribute to more effective use of gonadotrophins (FSH-P or PMSG) for superovulation. Polyovulation research has been applied to a 6-year, clinically healthy and sexually-known donor buffalo. On the 11th day of the sexual cycle, the superovulation protocol was applied. PMSG was used in the 2500 IU dose. At the onset of oestrus, a number of 8 pre-ovulatory follicles have been identified. The monitoring of follicular evolution until ovulation was performed ultrasonographically. Of the 8 follicles increased 3 developed on the left ovary and 5 on the right ovary. After 7 days of ovulation have been identified six corpora lutea (2 ovary left and 4 right).*

**Key words:** *buffalo, oestrus, poliovulation, MOET.*

### **Introduction**

Buffalo usually attain puberty when they reach about 60% of their adult body weight (250 to 400) kg, but the age at which they attain puberty can be highly variable, ranging from 18 to 46 months (Jainudeen and Hafez, 1993). The factors that influence this are genotype, nutrition, management and climate. It could be attained under optimized conditions at 15 to 18 months in river buffalo and 21 to 24 months in swamp buffalo (Borghese, 2005).

High individual variability in follicular recruitment and hence in the number of embryos produced is a major factor limiting the application of reproductive technologies in buffalo. Therefore, the identification of reliable markers to select embryo donors is critical to enroll buffaloes in embryo production programs. Better understanding of factors involved in follicular growth is also necessary to improve the response to superovulation in this species.

Buffalo have a relatively high efficiency of feed utilization compared with cattle, which has important implications for resource use and the environment (Campanile G et al 2014, Neglia G 2014).

Reproductive function in female river buffalo is closely linked to the environment. In equatorial areas and tropical regions, reproduction is more strongly influenced by nutrition. In the Mediterranean and higher latitudes, it is some distinct annual cycles in day length, female river buffalo are responsive to photoperiod. In Southern Europe (Romania), buffalo behave as a short-day breeder and show an increase in reproductive function during the summer-autumn transition (decreasing day length) and a decrease in reproductive function during the winter-spring transition

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(increasing day length). The latter period is characterized by reduced ovarian activity and function (Campanile G, 2010).

Similar to ruminants, buffalo have ovarian follicular waves and two waves in each estrous cycle is the most common pattern. Ovulation occurs between 26 and 33 hours after the LH surge (Baruselli P 1997, Presicce GA 2005, Neglia G, 2007, Seren E 1995, Porto-Filho RM 1999).

The ovaries of post-pubertal buffalo heifers have a reservoir of only 10,000–20,000 primordial follicles (Danell, 1987) compared with over 100,000 in cattle. The mature ovaries are smaller than in cattle, weighing around 2.5 g when inactive and 4 g when active, with fewer tertiary follicles

Early studies on synchronization of time of oestrus in buffalo were based on those developed for cattle, aimed at either inducing premature luteolysis using prostaglandins or prolonging the luteal phase using progestagens (Perera, B. 2008.).

Follicular wave dynamics on the outcome of these procedures (De Rensis, F 2007) have prompted studies aimed at manipulating follicular development to achieve greater oestrous synchrony and improved fertility.

Gimenes et al. (2007) reported that follicles in Murrah×Mediterranean buffalo heifers acquired the capacity to ovulate in response to exogenous LH when they reach a diameter of 8.5–10.0mm. On the other hand follicles from multiparous Mediterranean cows with diameters ranging from 4.2 to 13.0mm ovulated when treated with hCG or GnRH agonist. In the latter studies, follicles ranging from 4.2 to 13.0mm did not consistently ovulate after treatment with hCG or GnRH agonist suggesting that size does not necessarily reflect stage of follicle development or functionality.

In buffaloes undergoing regular oestrous cycles dominant follicles attain a size of 13–15mm before ovulation (Neglia et al., 2007) and this could be interpreted to suggest that, under normal conditions, follicles in buffaloes acquire the capacity to ovulate at around 5–8 mm but must undergo further development in order to secrete sufficient oestradiol for a positive feedback action to induce the pre-ovulatory LH surge.

**Follicular superstimulation.** Follicular superstimulation is most successful when the initiation of exogenous FSH treatment is timed to coincide with the endogenous wave-inducing surge of FSH and emergence of the second follicular wave. This is difficult to achieve in randomly cycling buffaloes because of the rather large variation in duration of the first follicular wave. A second requirement in follicular superstimulation is the ability to control the time of ovulation in order to implement fixed-time AI. The injection of GnRH or porcine LH 24 h after the last treatment with FSH results in predictable and synchronous ovulation in buffaloes which removes the need for oestrous detection for AI (Baruselli et al., 2002).

In a superstimulation protocol that utilised a GnRH agonist to downregulate the anterior pituitary in buffaloes, the injection of porcine LH 24 h after the last injection of FSH is associated with greater ovulation rate, fertilisation and embryo recovery compared with injection of LH 12 h after the last FSH (Zicarelli, L. 2000).

The production of sufficient number of viable embryos through the use of gonadotropins for embryo transfer and multiple birth has been a subject of interest in buffaloes. In particular, follicle stimulating hormone (FSH) and pregnant mare serum gonadotropin (PMSG) have become the primary superovulatory drugs for donor buffalo cows. FSH is usually given within a 4- to 5-day period at midcycle as in cattle through a series of injections so that follicles are recruited immediately prior to lysis of the existing corpus luteum. A total dose of 40mg to 50mg is given to elicit optimal ovarian stimulation. The series of injections is required because of its short half-life in the circulation. The PMSG is used as a single injection of 2000-3000 IU.

Comparatively, FSH stimulation of ovaries results in more ovulation and recovery of embryos of better quality than with PMSG (13). A luteolytic dose of prostaglandin F 2 alpha (PGF 2 α) is usually given 48 h after initiation of the treatment. In all studies published to date, the superovulatory responses have varied, with low fertilization rates.

Rations with a higher level of protein influence the animal's level of luteinizing hormone (LH) and response to gonadotropin releasing hormone (GnRH), which would eventually affect the ovulation response of the donor. In addition, the season during superovulation treatment influences the rate of ovulation.

The use of either pure FSH or a controlled FSH -LH mixture plus GnRH and its analogues and/or an anti-PMSG, if PMSG is to be used, may improve the donor ovulation response.

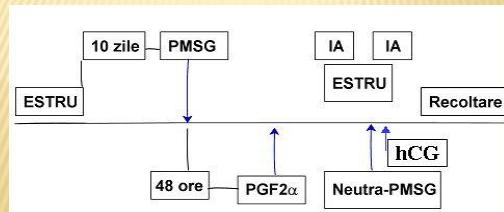
### Results, material and methods:

Polyovulation research has been applied to a 6-year, clinically healthy and sexually-known donor buffalo.

On the 11th day of the sexual cycle, the superovulation protocol was applied. PMSG was used in the 2500 IU dose. At the onset of oestrus, a number of 8 pre-ovulatory follicles have been identified. The monitoring of follicular evolution until ovulation was performed ultrasonographically. Of the 8 follicles increased 3 developed on the left ovary and 5 on the right ovary. After 7 days of ovulation have been identified six corpora lutea (2 ovary left and 4 right).



- ↗ (PMSG) Folligon - 2500 UI, i.m. Administered in a 11 th day of cycle.
- ↗ - 48 h after, prostaglandin F2 $\alpha$  - 15 mg (Prosolvon)
- ↗ 48-72h - I.A. 2x12 ore
- ↗ two hours ahead IA - PMSG (1500 UI) (Chorulon)



**Fig. 1** Schedule the polyovulatory treatment in buffalo

**Table 1.**

#### Poliovulation treatment and his effect

1	<i>PMSG (Folligon) On the 11th day of the sexual cycle</i>	2500 UI, im	
2	Poliovulation effect	Left ovary	Right ovary
3	8 ovarian follicles	3	5
4	6 corpus luteum	2	4



**Fig. 2 .** Buffalo, right ovary. Five follicles (F) identified by ultrasound, after 72 hours from the administration of PMSG



**Fig. 3 .** Buffalo, right ovary. Corpora lutea (CL), identified by ultrasound after 7 days from the administration of HCG

## Discussion

A review of studies using various treatment protocols (Borghese, A. 2005), shows that CR achieved with prostaglandins either alone or in combination with GnRH ranged from 7 to 56%, while CR following the use of a progesterone releasing devices either alone or in combination with eCG, and in some cases further supplemented with hCG or GnRH, ranged from 8 to 64%. The ‘Ovsynch’ protocol (GnRH followed by prostaglandin 7 days later and a second GnRH 2 days later) has been used successfully in buffalo, with synchronization of time of ovulation in 70–90% and CR of 33–60%.

In most studies the success rate was lower when treatment was done during the periods of low breeding activity or during seasonal anoestrus, and various modified protocols have been tried to overcome these problems. The two most effective approaches appear to be the Ovsynch protocol supplemented by administration of progesterone for 7 days between the first GnRH and prostaglandin treatments, and progesterone based regimens of 10–14 days with either GnRH or oestradiol treatment at the time of progesterone implant insertion and prostaglandin plus eCG treatment at implant removal (De Rensis and López-Gatius, 2007).

In addition to the type of protocol selected, the following factors must also be addressed when using any regimen in buffalo (Perera, 2008): (a) selection of animals that are in good body condition score and free from disease; (b) minimize stress during the treatment administration and AI, especially under tropical conditions, when animals may be herded together, tethered or moved to other locations; and (c) where seasonal differences exist, scheduling treatments for the more favourable periods or during the peak of the breeding season when the majority of animals are likely to be having oestrous cycles.

Is a worldwide increasing interest in largescale in vitro production of buffalo embryos to improve genetic progress through the maternal lineage. Indeed, the Ovum pick-up (OPU) and the in vitro embryo production (IVEP) are currently the most competitive technologies to produce transferable embryos (TE) over the long term.

Therefore, this procedure is a valid alternative to multiple ovulation/embryo transfer (MOET) programs because of the low response to hormonal stimulation, the poor embryo recovery, and the impossibility to repeat continuously the MOET treatments over the long-term. Indeed, multiple ovulations can be induced only in cyclic animals, whereas it is likely that buffalo cows enter seasonal anestrus (Zicarelli L 1997).

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# **Epidemiology of echinococcosis and changes in some chimal indicies of muscular tissue and liver of cattle in the Republic of Moldova**

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## **Abstract**

*The study of the parasitological invasions with *Echinococcus granulosus* in about 8000 cattle have been carried out in different time periods (1986-1987, 2014-2015) in private farms, family household and specialized in milk and beef production farming sectors from the Republic of Moldova. The results of the parasitological research carried out in 1986-1987 have revealed the 33.6% incidence of echinococcosis in adult cattle from farming sectors, 60.6% incidence in farms and 76.5% in cattle from family household (mean 56.9%); the incidence related to bull-calves population (of 23-25 months old) were 8.3%, 17.7% and 21.1% correspondingly (mean 15.7%). In the period 2014-2015, after the restructuring carried out in the livestock, the results show that adult cattle from farms were infested at 74.6%, in individual sector - in 83.7% of cases (mean 79.2%) and young cattle (23-25 months old) - in 31.2% and 37.4% cases correspondingly (mean 34.3%). Compared to 1986-1987 the level of infestation of adult bovines increased, in average, with 22.3% and of young cattle with 18.6% which prove the existence of various outbreaks of parasitic agents (definitive hosts) and lack or irregular conducting of dehelmentization. It was determined that the infested with echinococcosis cattle produce less meat and eatable by-products. Moreover, the content of some vitamins (A, E, B<sub>1</sub>, B<sub>2</sub>, C), micro-, macroelements (Ca, Mg, Na, K, Fe, P), chemical indicies (humidity, dry solid matter, proteins, fat, mineral saults) in muscular and liver tussues proved to be considerably changed influencing considerably the animal products' nutrition value.*

**Key words:** cattle, *Echinococcus granulosus*, vitamines, micro-, macroelements in meat and liver, nutrition value.

## **Introduction**

In the last 2-3 decades in connection with land allotment, reorganization of breeding, creation of many medium and small farms, redeploying of large numbers of animals from complexes to private households radical changes of parasitic fauna occurred. Cattle that were kept indoors, moving to different grazing anthropogenic stations also entered in nature reserves, serving as sources of transmission of parasitic agents to wild animals. The limited space dislocation and grazing of different species and various ages also contributed at increasing of parasitic fauna.

Spassky A., Andreiko O. and Selivanova N. in 1962 studied the spreading of echinococcosis in the Republic of Moldova and have established that the invasion extensivity in cattle in that period constituted 86.0% and in swine – 66.61%.

Hirik M. and Pomirko T. (1975) mention that cattle slaughtered in meat processing factories in the republic in 1964 were infested with echinococcus in 16.6% of cases and slaughtered cattle in 1973 - in 24.2% of cases. As the authors remark the invasion extensivity grew every year. If in the sixties there were established only few hydatid cysts in the liver and lungs, in the early seventies the invasion intensity increased, and they were detected in kidneys, spleen, and other organs. In most cases, the lungs and liver, because of infestation were destroyed.

Zgordan E.S. (1974) mention that in cattle 38 species of helminths were revealed. A uniformity was noted during their infestation with cestodes: in southern zone – 22.4%, central – 21.5% and in northern one – 23.3%.

The aim of the scientific research was to determine, in dynamics, the level of invasion with echinococcosis of the cattle depending on the age and maintenance techniques, as well as their impact on some chemical indexes, content of vitamins (A, E, B<sub>1</sub>, B<sub>2</sub>, C), micro- and macroelements (Ca, Mg, Na, K, Fe, P) in the muscular tissue and liver.

### Materials and methods

The parasitological research have been carried out in about 8 thousand of cattle of different age purchased from the private and public households with various maintenance technologies and from different regions of Moldova and slaughtered in the meat processing plant during 1986-1987, 2014-2015.

The content of some vitamins (A, E, B<sub>1</sub>, B<sub>2</sub>, C), micro-, macroelements (Ca, Mg, Na, K, Fe, P), chemical indices (humidity, dry solid matter, proteins, fat, ashes) in muscular and liver was studied. The content of micro- and macroelements have been determined by use of the photoelectric colorimetry methods, and the composition of the vitamins have been defined using the spectrophotometric methods. In view of this 30 samples of muscular tissue as well as liver have been collected from healthy cattle and cattle infested with echinococcosis.

### Results and discussions

The reforms realized in the animal production field of the Republic of Moldova in the last 20-30 years have lead to quantitative changes in the population of livestock: this have been reduced 2.5-3 times as compared to 1987. The ratio of the animals from the private sector by comparison to the public one considerably increases. Given that in 1987 the number of cattle in the private sector accounted up to 14.4% of the total number of the livestock population then already in 2015 this was increased up to 88.8% (table 1).

**Table 1.** Ratio between cattle population from household sector and private farms in the Republic of Moldova during 1987, 2015

Maintenance technology	1987	2015
Public sector, %	85.6	11.2
Private sector, %	14.4	88.8

There were described about 1200 species of parasites in the Republic of Moldova and over 100 are pathogenic for animals. Due to the fact that the animal fauna of Republic of Moldova is reach and the climate facilitates the development of various species of parasites, especially of helminthes, their study is essential for the monitoring of the sanitary, veterinary and epidemiological situation (Toderaş I., Vladimirov M., Neculiseanu Z., 2007).

Thus, in order to organize a complex program of measures for prophylaxis and treatment of parasitic invasions in animals its necessary, besides knowledge on etiology, pathogenesis, clinical status and treatment measures, to have data on the level of spreading of the various species of parasites related to animal age, its maintenance technologies, seasons etc. In this regard, the parasitological studies carried out in dynamics are of great importance.

The study of echinococcosis spreading in cattle have been carried out in different periods (1986-1987, 2014-2015) and geographic regions of the Republic of Moldova, in the specialized farms households, in the private sector and the complexes.

Parasitology research results obtained in 1986-1987 demonstrates that echinococcosis in adult bovine and young cattle is most commonly diagnosed in the individual sector (76.5%, 21.1%) and farms (60.6%, 17.7 %) (table 2).

Parasitological research results on echinococcosis extensivity in adult cattle (4-6 y.o.) and young cattle (23-25 months) purchased from various parts of the country and slaughtered in the period from 2014 to 2015, after restructuring carried out in the livestock sector, reveals that adult cattle were infested in 74.6% of cases in farms and in 83.7% of cases in the private sector, while young cattle respectively in 31.2% and 37.4% of cases (table 3).

**Table 2.** Level of infestation in adult cattle (4-6 y.o.) and in young cattle (23-25 months) depending on different maintenance technologies during 1986-1987

Age animals	Maintenance technology			
	Complexes, %	Farms, %	Private sector, %	Mean, %
Adult cattle (4-6 y.o.)	33.6	60.6	76.5	56.9
Bulls (23-25 months)	8.3	17.7	21.1	15.7

**Table 3.** Level of infestation in adult cattle (4-6 y.o.) and of young cattle (23-25 months) with echinococcus in 2014-2015

Age animals	Maintenance technology		
	Farms, %	Private sector, %	Mean, %
Adult cattle (4-6 y.o.)	74.6	83.7	79.2
Young cattle (23-25 months)	31.2	37.4	34.3

The level of infestation of adult bovines increased in average, with 22.3% and 18.6% bulls, compared to 1986-1987 and demonstrate the existence of various outbreaks of parasitic agents (definitive hosts) or irregular dehelminization.

Echinococcosis play a leading role in the structure of parasitic diseases in animals. It also provoke a higher risk of transmitting parasitic diseases to humans, as well as reducing the quality of animal products. After analyzing the number of cases of humans surgical operations for Echinococcosis/ Hydatidosis in Moldova during 1980-2016, we have to mention that it increased in the last decades (table 4).

While performing the sanitary and veterinary expertise of carcasses and organs of the animals it is recommended to determine (in regard to the intensity of invasion with echinococcosis) three levels of infestation: *low*, *medium* and *high*.

*Low infestation* occurs when in the animal is affected one third of the organ (liver, lung) with sporadic hydatids. In such cases there are no changes in the organ, with the exception of the destructive cells around hydatid cyst. Low infestation is more frequent established in young cattle, up to 2 years old (20-22%);

Medium infestation occurs when no more than half the organ is affected and several hydatid cysts are established (3-5) on the surface of the organ and inside it. Their size varies between 5-10 cm and parenchymal organ changes are observed, mainly in the hydatid cyst. This index is necessary to consider at sanitary-veterinary expertise of organs. Medium infestation is frequently determined in younger cattle of 2-4 years old (26-29%);

*High infestation* occurs where 2/3 or the whole organ is affected. On its surface or inside the organ there are multiple hydatid cysts of various sizes. Adult animals are affected more frequently (30-32%).

Thus, in slaughtered animals the low and medium intensity was determined more frequently.

**Table 4.** Number of (occasional) cases of surgical operations due to Echinococcosis/Hydatidosis in humans in the Republic of Moldova in 1980-2016 (Lungu V. et.al., 2016)

Years	Surgery cases	Years	Surgery cases	Year	Surgery cases
1980	64	1993	144	2006	135
1981	56	1994	183	2007	209
1982	49	1995	195	2008	140/12
1983	94	1996	188	2009	131/22
1984	96	1997	169	2010	167/19
1985	99	1998	215	2011	99/10
1986	105	1999	178	2012	118/9
1987	145	2000	175	2013	125/13
1988	156	2001	203	2014	88/22
1989	189	2002	228	2015	62/10
1990	195	2003	233	2016	55/4
1991	196	2004	200		
1992	149	2005	162		

Note: *nominator*- number of cases in adults; *denominator* - number of cases in children

There was determined the content of the vitamins A, E, B<sub>1</sub>, B<sub>2</sub>, C and micro- and macroelements - Ca, Mg, Na, K, Fe, P in order to establish the nutrition value of muscular tissue and liver in cattle infested with echinococcosis (table 5 and 6).

It was established that in infested cattle the content of vitamins A and E in liver decreased by about 3 times, of B<sub>1</sub> and B<sub>2</sub> - by 1.12 times and of C - by 1.59 times, of iron - by 2.64 times and of phosphorus - by 2 times, while of calcium increased by 3.42 times, of sodium - by 4.81 times, of magnesium – by 1.19 and of potassium - by 1.33 times in comparison to the non-infested animals.

**Table 5.** The content of vitamins in liver and tissues muscular in cattle infested with echinococcosis (mkg/g)

Studied material	A	E	B <sub>1</sub>	B <sub>2</sub>	C
<b>Liver:</b>					
Healthy	0.14	0.35	1.16	1.23	24.5
Infested	0.05	0.12	1.04	1.10	15.4
<b>Muscular tissue:</b>					
Healthy	0.11	0.20	1.17	1.47	16.8
Infested	0.03	0.14	1.00	1.10	4.0

In meat the content of vitamin A was 3.67 times lower, of E – 1.43 times lower, of B<sub>1</sub> - 1.17, of B<sub>2</sub> - 1.34, of C – 4.20, of calcium – 1.69 times lower, while of magnesium was 1.39 times higher, of sodium – 1.53, of potassium - 1.34, of iron – 3.26 and of phosphorus – 1.14 times higher than in healthy cattle.

**Table 6.** The content of micro- and macroelements in liver and in tissue muscular infested with echinococcosis cattle (*g/100 g of mineral substances*)

Researcher material	Calcium	Magnesium	Sodium	Potassium	Ferrum, (mg/100g)	Phosphorus, (%)
<b>Liver:</b>						
Healthy	1.17	1.21	1.30	9.38	752.50	2.50
Infested	4.00	1.44	6.25	12.50	285.00	1.25
<b>Muscular tissue:</b>						
Healthy	1.23	1.12	1.18	10.75	86.00	1.05
Infested	0.73	1.56	1.80	14.38	280.00	1.20

Echinococcosis in cattle leads to lower yields of meat and comestible by-products (table 7).

The obtained results indicate that in young cattle infected with echinococcus the content of meat has decreased by 10.6%, of fat - 10.1%, of by-products: liver - 45.5%, lungs - by 47.8% and in adult cattle, respectively decreased by 19.5%, 18.9%, 46.1% and 51.6%.

**Table 7.** Yield of meat and comestible by-products falling into first category obtained from infested with echinococcosis cattle.

Age category	Sample	The yield at cutting, kg			
		meat	fat	liver	lungs
Young cattle	Healthy	279.3	7.9	3.3	2.3
	Infested	249.7	7.1	1.8	1.2
Adult cattle	Healthy	222.5	9.5	3.9	3.1
	Infested	179.1	7.7	2.1	1.5

The veterinary expertise of carcasses and organs from cattle infected with various parasite agents that are not directly transmitted to humans, the affected organs partly or total, are validated, and the parties affected are destroyed and the non-affected parts a suitable for consumption as healthy ones without restrictions. The purpose of the research was to determine the content of chemical indices in meat and liver, depending on the intensity of infestation with echinococcus (table 8).

From the obtained results it follows that in cattle with low intensity of infestation with echinococcus the moisture content in meat increased by 1.8%, average - by 2.2%, those with high intensity of infestation - by 3.4% and in liver - 0.7%, 4.4% and 8.7% accordingly.

In cattle with low intensity of infestation the dry matter content in meat decreased by 5.0%, average - by 6.1%, with high intensity of infestation - 9.9% and in liver, respectively - by 1.66%, 10.27% and 16.89%.

The amount of protein in meat from cattle with low infestation intensity decreased with 0.5%, average - with 3.7% and those with high intensity of infestation with 4.6% and in liver, respectively – with 4.3 %, 7.3% and 14.7%.

In cattle with low intensity of infestation the fat content in meat decreased with 24.24%, average - 30.3% and high - with 42.42%, in liver, respectively - with 22.73%, 27.27% and 50.0%.

**Table 8.** The chemical composition of the meat and liver of the infested with echinococcosis cattle

Study sample	Intensity of invasion	Humidity	Dry substance	Proteins	Lipids	Mineral substances
Muscular tissue	Healthy	73,8±0,1	26,2±0,06	21,7±0,2	3,3±0,06	1,2±0,06
	Low	75,1±0,06	24,9±0,1	21,6±0,06	2,5±0,12	1,2±0,1
	Medium	75,4±0,12	24,6±0,06	20,9±0,06	2,3±0,06	1,0±0,1
	High	76,3±0,15	23,6±0,15	20,7±0,12	1,9±0,1	1,0±0,06
Liver	Healthy	69,8±0,1	30,2±0,2	23,2±0,12	4,4±0,23	1,4±0,12
	Low	70,3±0,15	29,7±0,2	22,2±0,1	3,4±0,1	1,2±0,1
	Medium	72,9±0,1	27,1±0,15	21,5±0,06	3,2±0,17	1,2±0,06
	High	75,9±0,1	25,1±0,1	19,8±0,15	2,2±0,1	1,1±0,06

In cattle with low intensity of infestation the quantity of mineral substances in the meat wasn't reduced, and in those with medium and high intensity of infestation was reduced with 16.7%. In the liver in cattle with low and medium intensity of infestation it decreased with 14.3% and those with high intensity - with 21.4%.

Thus, the results of parasitological studies after the slaughtering the animals in slaughter houses prove that the echinococcosis in cattle of different age and as of different maintenance technologies is frequently recorded and reveal the irregular conducting of dehelminization and in some farms – its total absence. In infected animals it provokes significant decrease in gaining daily weight, modification in chemical composition, vitamins, micro- and macroelements in meat and comestible by-products and reduces their nutrition value. Thence, it is necessary to improve the system of evaluation of animal status, meat and comestible by-products originated from the infested animals.

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## **Conclusions**

1. It was established a high level of infestation of cattle with echinococcus, across geographical zone of growth and maintenance of their technology, constituting 79.2% of cases in adult bovine and 34.3% in young cattle (23-25 months).
2. The high level of infestation with echinococcus is possible, probably due to continuous contact with the definitive host and lack of dehelminthization or its irregular conducting.
3. In cattle infected with echinococcus there are changed the content of vitamins (A, E, B1, B2, C), micro- and macro-elements (Ca, Mg, Na, K, Fe, P) and chemical indices (moisture, dry matter, protein, lipids, minerals) in muscle tissue and liver that negatively affects considerably the nutritional value.
4. When processing the meat and edible by-products it is necessary to consider the intensity of cattle infestation with echinococcus. The meat and edible by-products without echinococcus should be used depending on nutritional value: at low infestation – without restriction, at medium infestation – only industrial processing (manufacture of sausages, canning etc.), high infestation - industrial processing only after bacteriological research.
5. Payment to economic agents in the realization of live animals, carcasses and edible by-products, to be carried out depending on the level of infestation with parasitic agents.

## **Acknowledgements**

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# The functional status, resistance and adaptive capacities of the calves being affected by combined stressors during their early postnatal ontogenesis

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## Abstract

The experimental studies revealed and analyzed the dynamics, protein and saline metabolism, resistance to stress factors and adaptive capacities (total proteins and protein fractions, urea, macro elements Ca, P, Na, K, content of glucose, bactericide activity, cortisol concentration) of calves in their early postnatal ontogenesis under the combined influence of stresogen thermal maintenance factor, noise and parasitic factors. There were established that the fluctuations of the researched physiological parameters have the phasic character. The tested indices, in major cases were quantitatively lower in the group of animals affected by the combined stressor factors. Two ascends in changing of the indices have been observed: at the 7-8-th and at the 25-30-th days at birth that coincided with critical development periods: immunodeficiency, depression of the stresogen reaction, depression of dominance and retardation. The positive effect has been registered also upon stress resistance and adaptation capacities of the organism.

**Key words:** cattle, resistance and adaptive capacities, parasitar factors.

## Introduction

The environmental factors (optimal, stressors, extremal) have an effect on organism in multiple situations of combination of such and the impact of their application depends on its nature and intensity (Furduy F. I. and others, 1982, 1985, 1992).

According to data reflected in scientific literature, the complex of factors applied simultaneously in most cases cause aggravation of the deviations of the functional status of the organism, compared to their separated application, especially in the critical periods of early ontogenesis (Dobrovols'kiy L. A., 1982; Furduy F. I. and others, 1985; Erhan D. C. etc., 2007; Pavaliuc P. P. etc., 2012).

At the same time, the influence of the simultaneous combined factors can be favorable and the effects of the applied combined environmental factors may be of these three types: additive, synergistic and antagonistic. This classification reflects the essence of the effects that could be observed while factors of various nature affect the organism (Antipijv V. V. and others, 1980; Furduy F. I. and others, 1985; Erhan D., Pavaliuc P., Rusu S., 2007; Rusu S., 2012).

The specialized scientific literature lack data on effects of the combined action of the maintenance stressor factors on the organism of calves during their early postnatal ontogenesis. The presented study has been targeting on establishment of the functional status, resistance and adaptive capacities of calves in their early postnatal ontogenesis while affected with stressor thermal maintenance factor being combined with other stressors such as excessive noise and parasitic factors.

## Materials and methods

The research has been undertaken on calves of Black-and-White Holland race during their early postnatal ontogenesis and under controllable conditions. On the leash, similarly to the

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conditions of households where intensive technologies usually applied, the temperature, noise and parasitic factors are the most predominant one that provoke stress in animals. The thermal factor of the stressful intensity combined with a noise of 70-80 db and parasitic agents (*Eimeria spp.*, *Strongyloides papillosus*) have been applied to calves following the research purpose.

The animals have been placed in the climacteric camera allowing them to adapt to the new conditions during 40-60 minutes, and afterwards the temperature have been decreased up to 5°C. The stressing temperature combined with noise has been applied on 3-rd, 8-th, 15-th, 20-th, 25-th and 30-th days at birth.

The blood has been collected from the adapted animals just before and after applying combined stimuli. The traditional methods of data processing have been used for the analysis of the collected material. The following blood indices have been researched: the content of total proteins, protein particles, glucose, alkaline reserve, Ca, P, Na, K-levels, cortisol, bactericide activity as well as the growth rate values. The values of the physiological indices studied before applying combined stimuli have been serving as the control set (control group).

## Results and discussions

The previous scientific studies revealed the specifics of influence of the stressing thermal maintenance factor on the functional status, resistance and adaptive capacities of calves during their early postnatal ontogenesis (Pavaliuc P., Erhan D., Rusu S. et al., 2012).

An special interest represents the results of the research when stressing temperature is applied along with another such widespread in households' factor as noise. The carried out experiments allowed studying the functional status, resistance and adaptive capacities of the organism of calves during their early postnatal ontogenesis while affected by combined stressor maintenance factors such as temperature and noise. The obtained results are presented in Table 1.

The analysis of obtained data shows that the complex effect of thermal and noise factors at 3-rd and 8-th days at birth, the concentration of total proteins have not been affected considerably compared to its indices before the influence of stressor factors, so that only at the 15-th day this have been increased by 6,5%, and at the 20-rth - by 8,5%, at the 25-th day- by 9,9%, and at 30-th day at birth - by 19%, as compared to the control group. There should be mentioned the relatively stable character of the concentration of blood proteins and its non-essential increase after applying combined stressors under maintenance condition as compared to the control group.

The analysis of the concentration of the protein particles revealed that the concentration of blood albumins has been decreased by 14,0% and by 10,4% correspondingly after applying combined stressors at 8-th and 15-th days at birth as compared to the control group. Just after the 20-th day the increase (by 38,4%) has been observed that reached the maximum level at the 25-th day as compared to the control group. While applying the stressor factors at the 8th day of birth the decrease of the concentration of  $\alpha$ -globulins by 2,9 times, and at 25-th day - by 2,3 times has been noted. In this way, the dynamics of changing's in protein particles in calves during their early postnatal ontogenesis is characterized by the decrease of concentration of  $\alpha$ -globulins during its first period. Further on, after 15-th day one can observe an increase, especially at the 30-th day. An increase in concentration of  $\beta$ - and  $\gamma$ -globulins at 8-th day, especially of  $\beta$ -globulins (by 1,9 times) have been observed, less marked for  $\gamma$ -globulins (by 1,2 times). Under the influence of combined maintenance stressor factors, the level of  $\beta$ -globulins in blood at the 25-th day at birth has decreased by 1,3 times as compared to its values in control group, and the level of  $\gamma$ -globulins remained approximately at the same level as it was before applying the stressor factors.

While applying the combined stimuli the concentration of urea have been increased prominently only at 25-th and 30-th days (by 49,3 and 20,3% correspondingly). The similar

increase has been observed also before applying these. This increase could be explained by the introducing after 20-th day in the animal ration of the complex food (hay, haulage).

This being said, one can observe the tendency of the increase of the level of the total proteins starting with 15-th day and the level of urea – starting with 25-th day. The limits of evaluation of these indices have been relatively stable. The level of changing's in the protein particles to a great extent has been correlated with the level of changing's in the total proteins.

Another important physiological indices that characterize the functional status of the organism is the level of saline metabolism that could be explained by determining the concentration of macro elements (Ca, P, Na, K) in the blood, and their correlations (Ca:P; Na:K).

**Table 1.** The dynamics of the protein metabolism indices researched during early postnatal ontogenesis of calves affected in maintenance conditions by combined impulsive thermal and noise factors (n=10 animals)

Age (days)	Total proteins, g/1	Protein particles, %				Urea, mmol/l	
		albumins	globulins				
			$\alpha$ -	$\beta$ -	$\gamma$ -		
<b>Before the influence of stressor factors</b>							
3	59.2±2.49	55.3±2.64	10.5±0.31	14.5±0.39	19.7±0.56	1.72±0.08	
8	58.3±2.50	622±3.08	7.2±0.30	14.3±0.42	16.3±0.47	2.15±0.11	
15	51.8±2.41	72.8±3.01	6.3±0.29	11.5±0.38	9.4±0.26	2.00±0.11	
20	52.1±2.48	61.3±2.74	15.8±0.43	14.9±0.43	8.0±0.21	2.04±0.11	
35	52.5±2.51	50.0±2.06	26.7±0.97	18.3±0.45	5.0±0.19	2.01±0.09	
30	51.9±2.43	65.8±2.53	15.9±0.54	9.1±0.27	9.2±0.24	3.16±0.12	
<b>After the influence of stressor factors</b>							
3	60.1±2.63	52.1±2.41	3.7±0.12	25.2±0.41	19.0±0.29	1.83±0.09	
8	58.3±2.51	53.5±2.47	2.5±0.11	25.4±0.44	18.6±0.29	2.30±0.11	
15	55.4±2.48	65.2±2.63	6.5±0.18	20.7±0.42	7.6±0.28	1.87±0.09	
20	57.0±2.50	66.9±2.71	8.7±0.21	18.1±0.38	6.3±0.21	2.43±0.11	
25	58.3±2.51	69.2±2.84	11.5±0.25	13.6±0.36	5.7±0.19	3.00±0.15	
30	54.1±2.61	66.3±2.68	13.0±0.39	11.9±0.32	8.8±0.24	3.80±0.21	

P ≤ 0,05

The dynamics of the changing's in the concentration of these elements in the blood of calves during their early postnatal ontogenesis before and after applying combined stressor factors – temperature and noise under maintenance conditions-is presented in Table 2.

Similarly to situation of concentration of Ca upon applying stressor factors, the concentration of P observed an insignificant tendency of changing's that still falls into the norm limits (1,45-1,90 mmol/l). While applying the stressor factors, the correlations of these two elements have been slightly increased as compared before applying these, yet again falling into the norm limits.

The data from Table 2 reveal that the level of Na in blood of the calves affected by combined stressing thermal and noise factors have been increasing from the 3-nd to 25-th days, and its decrease have been observed in the ulterior periods of research. The content of K in blood in the

same circumstances remains almost unchanged. Thus, the obtained data show the stable character of Na' and especially of K' metabolism that could be observed from its values' dynamics.

The data from Table 2 shows that the concentration of Ca during all period of applying the combined stimuli have been higher as compared to the control group and this tendency, starting with 8-th day have been approximately corresponding to 0,2 mmol/l. At the same time, there should be mentioned the decrease in dynamics of levels of Ca, and an increase of its level at 30-th day in both groups.

**Table 2.** The dynamics of the saline metabolism indices in calves during their early postnatal period at influence of impulsive thermal and noise stressors (n=10 animals)

Age (days)	Ca, mmol/l	P, mmol/l	Ca:P	Na, mmol/l	K, mmol/l	Na:K
Before the influence of stressor factors						
3	2.80±0.12	1.87±0.10	1.49±0.85	155.41±5.01	6.62±0.33	23.55±1.2
8	2.60±0.11	2.07±1.11	1.26±0.78	158.64±4.63	6.83±0.29	23.32±1.2
15	2.55±0.11	1.62±0.14	1.57±0.91	152.40±5.03	5.80±0.21	26.28±1.2
20	2.47±0.90	1.58±0.08	1.56±0.72	153.3±4.94	6.44±0.27	23.95±1.2
25	2.40±0.80	1.52±0.06	1.58±0.84	154.61±5.00	7.03±0.31	22.09±1.2
30	2.60±0.11	1.62±0.11	1.60±0.86	150.0±5.01	6.41±0.38	23.47±1.2
After the influence of stressor factors						
3	2.83±0.12	1.90±0.12	1.49±0.90	132.73±5.20	6.33±0.28	21.06±1.1
8	2.80±0.12	1.94±0.11	1.44±0.75	139.22±5.00	6.42±0.31	21.75±1.2
15	2.75±0.11	1.74±0.09	1.58±0.83	150.61±5.11	5.71±0.17	26.42±1.3
20	2.68±0.11	1.70±0.10	1.58±0.91	152.10±4.98	6.30±0.23	22.56±1.2
25	2.60±0.90	1.64±0.08	1.59±0.87	153.40±4.57	6.84±0.30	22.56±1.2
30	2.85±0.12	1.68±0.09	1.70±0.89	147.41±4.89	6.21±0.25	23.77±1.2

P ≤ 0,05

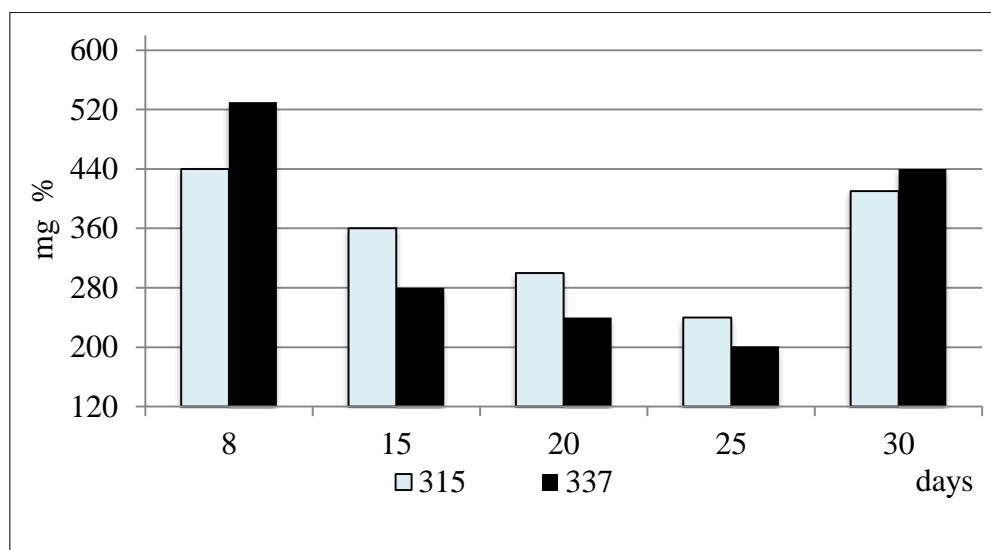
The conducted experimental studies allowed also observing the dynamics of the level of glucose and alkaline reserve in calves' blood during their early postnatal ontogenesis while applying combined stressing thermal and noise factors. The obtained data are presented in Figure 1 and 2.

The data from Figure 1 indicate the concentration of glucose in the blood of animals at influence of impulsive thermal and noise stressors during their early postnatal ontogenesis that varied and its dynamics could be characterized by the phasic aspects. Its maximum level has been observed at 8-th and 25-th days (6,66±0,24 and 5,55±0,18 mmol/l).

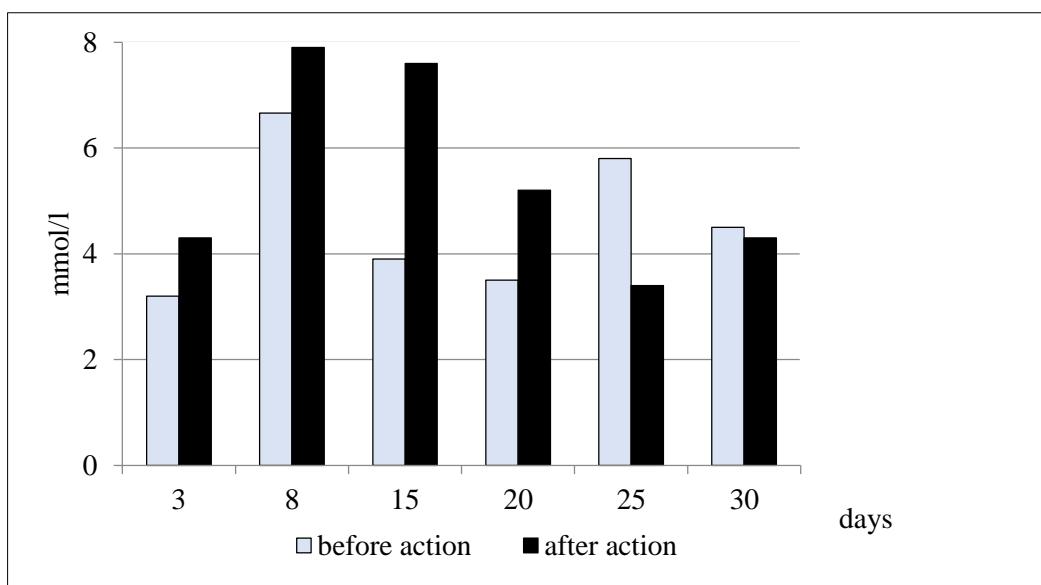
Under the influence of stressing factors the concentration of glucose have been observed as increasing one during all ontogenesis periods (especially at 15-th day – nearly 2 times) excepting the 25-th day at birth when its sharp decline (by 39,6%) has been observed. These two quite pronounced peaks of changing's have been noted at the 15-th and 25-th days as compared to other periods.

Similarly, the changing's in the dynamics of the alkaline reserve (Figure 2) had the phasic character and its increased level (before and after applying stressing factors) up to 8-th day

followed by a decrease of its concentration up to the 25-th day, and a phase of increasing values (on 30-th day). There is a reciprocal correlation of the level of alkaline reserve and level of glucose in the blood. While applying the combined stressing factors at 8-th day at birth the alkaline reserve has been increasing as compared to the control group by 9,1%, and at 15-th day it declined by 22,2%, at 25-th day – by 16,6%. Finally, at 25-th day it declined 2,6 times, if compared to the levels of the 8-th day.



**Figure 1.** The concentration of glucose in blood plasma of calves during their early postnatal ontogenesis at influence of impulsive thermal and noise stressors

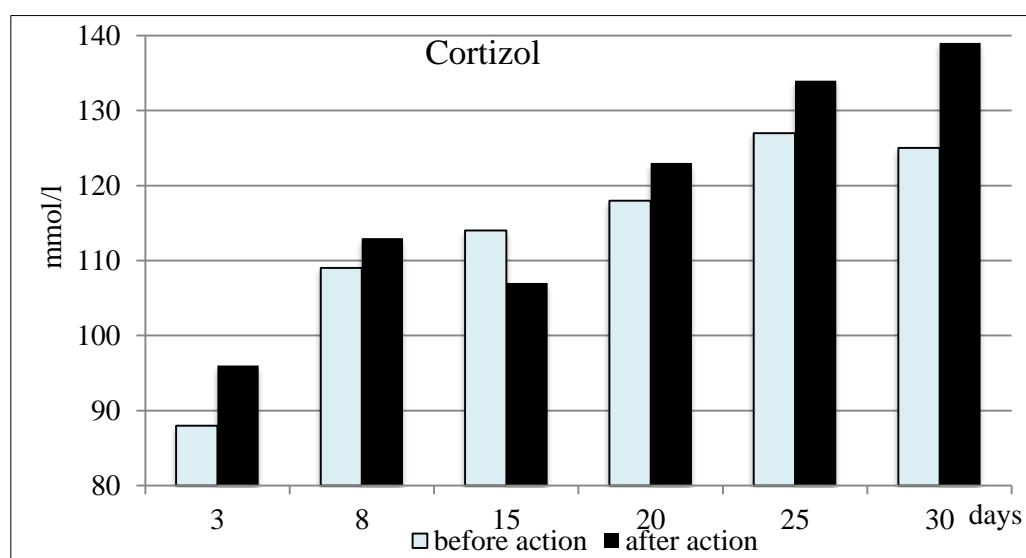


**Fig. 2.** The concentration of alkaline reserve of calves during their early postnatal ontogenesis at influence of impulsive thermal and noise stressors

In this way, the decrease in the level of glucose and alkaline reserve demonstrates the mobilization of the energetic resources in the organism as a response to the developed reaction to stressors. The muscular system became maturated, this period being a dominant one.

Some physiological indices that characterize the resistance of the organism to the stressing factors such as bactericide activity and cortisone concentration in blood have been also studied (Figures 3, 4).

The data from Figure 3 indicate that concentration of cortisone had a tendency of slight increase excepting the 15-th day when it decreased to the level of the control group. This could be explained by the fact that the major part of maternal immunoglobulin's have already disintegrated but the own system not yet matured (period of immunodeficiency). That is why the release of hormone is intensified and its quantity is increasing gradually corresponding to the process of maturation of suprarenal capsules determining the organism to adapt to the changed environmental conditions.



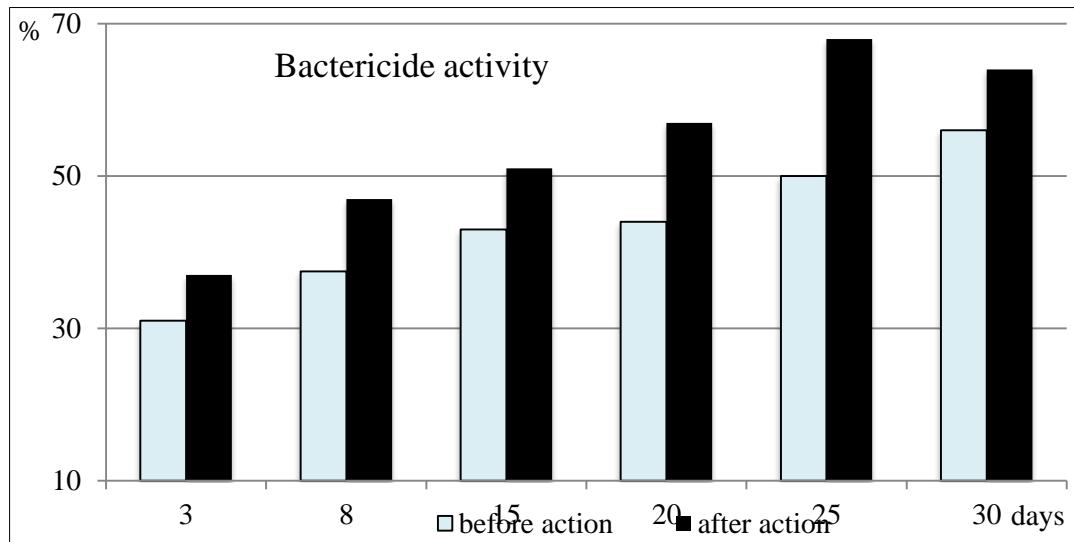
**Fig. 3.** The dynamics of cortisone concentration in blood of calves during their early postnatal ontogenesis at influence of combined thermal and noise stressing factors

The index that characterizes the stress, resistance and adaptive capacities of the organism to the action of the stressing factors also served the bactericide activity (Figure 4). The obtained data prove that the applying the combined stressing factors cause the increase of this index as compared to the control group, and this have been constant during all period of research constituting 23,9% in average. The biggest increase (by 36,7%) of bactericide activity, compared to the control group, have been registered at 25-th day.

The tempo of calves' growth during critical periods of early postnatal ontogenesis at influence of combined thermal and noise stressing factors have been slightly increased as compared to the control group that could be explained by the induced mobilization of plasticity and energy compensation reactions. This tempo is decreasing in the experimental group compared to the control one just after the critical periods of growth.

During previous studies the influence of stressing abiotic factors on the particularities of the functional status, resistance and adaptive capacities of the calves during their early postnatal ontogenesis have been researched as a separate issue (Pavaliuc P., Erhan D., Rusu S. et al., 2012).

Later on, 2 series of experiments have been carried out while testing in a separate ways the stressing factors in two groups of calves: 1) applying the combined abiotic stimuli (stressing temperature + excessive noise), the results of which are presented above; 2) control group, applying the biotic parasitic factor, by applying the combined abiotic and biotic stimuli (stressing temperature + excessive noise + parasitic factor). The obtained results are presented below in Table 3.



**Fig. 4.** The dynamics of bactericide activity in the blood of calves during their early postnatal ontogenesis at influence of combined thermal and noise stressing factors

**Table 3.** The indices of protein metabolism in calves during their early postnatal ontogenesis at influence of mono- and multifactorial stimuli (n=10 animals)

Age (days)	Total proteins, g/l	Protein particles, %			
		globulins		$\alpha$ -	$\beta$ -
		albumins			
<b>Control group</b>					
15	59.27±2.47	62.37±2.91	11.90±0.30	14.17±0.40	13.35±0.38
<b>After applying the parasitic factor</b>					
15	83.33±1.41	37.65±1.70	8.55±0.40	13.70±0.8	31.50±1.25
<b>After applying the combined stressing temperature, excessive noise and parasitic factors</b>					
15	68.21±2.40	50.18±1.89	9.58±0.19	16.98±0.33	10.89±0.26

P ≤ 0,05

The data presented in Table 3 indicate that the studied indices have been considerably changed in calves under the influence of the biotic parasitic as well as of the combination of abiotic and biotic stressors, as compared to the control group. The concentration of total proteins and protein particles' spectrum have been changing as follows: while applying the separate parasitic stressor – the content of albumins,  $\alpha$ - and  $\beta$ -globulins have been decreased by 39,6; 28,1 and 3,3% correspondingly while the content of total proteins and level of  $\gamma$ -globulins have been increased by 40,6% and by 2,4 times correspondingly.

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The spectrum of protein particles while applying the combined abiotic and biotic stressors has been changing in the following way: the content of albumins,  $\alpha$ - and  $\gamma$ -globulins have decreased by 19,5; 19,50 and 18,4% while the total proteins and the level of  $\beta$ -globulins have been increased by 15,1% and 19,8%. The analysis of the obtained results reveals that the concentration of total proteins and  $\gamma$ -globulins in calves from the group affected with combined thermal along with excessive noise and parasitic stressors, registered a decrease of 18,1% and by 2,9 times, and the concentration of albumins,  $\alpha$ - and  $\beta$ -globulins has increased by 33,3; 12,1 and 23,9% as compared to the group of animals affected with parasitic stressor only. This could be explained by the impact and way of stimuli effect on the organism of calves during their early postnatal ontogenesis.

In this way, two ascents of pronounced changes of the tested physiologic indices at 8-15-th and 25-30-th days at birth have revealed the coincidence with some critical periods of early postnatal ontogenesis—immunodeficiency, depression of the stressogen reaction, depression of dominance and retardation. As a conclusion, the registered changes had a phasic character.

### Conclusions

1. There were observed that the changes in the studied physiological indices (total proteins, protein particles, glucose, alkaline reserve, macro elements Ca, P, Na, K, cortisol, bactericide activity) while applying the combined temperature and noise stressing factors have a phasic character and have been influencing – by increasing or decreasing its values during early postnatal ontogenesis. The most evident changes have been noted at the 8-15-th days and 25-30 days at birth. These changes have corresponding with critical periods of ontogenesis: immunodeficiency, depression of the stressogen reaction, depression of dominance and retardation.
2. A tendency of increased bactericide activity and the level of cortisol in blood (with exception at the 15-th day) have been observed while applying the combined stressor factors, that proves the fact of some increased resistance level and adaptive capacity of the animal organisms.
3. There were observed that the impact of the combined abiotic and biotic factors (stressing temperature + excessive noise + parasitic agents) essential aggravates the metabolic processes in the organism of the calves as compared to these while applying mono factorial stressors.

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# **Infestation of amfibians from *Pelophylax Esculenta* (*Amphibia*) complex with the trematode species *Codoncephalus Urniger* (*Strigeida*) in Central Codrii in the Republic of Moldova**

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## **Abstract**

The paper presents data on amphibians' trematodes infestation from *Pelophylax esculenta* complex (*Amphibia, Anura*) in natural and anthropized ecosystems of Central Codri forest from the Republic of Moldova. The helminthological researches of amphibians were accomplished for the first time in the Republic of Moldova. As result, the infestation of *Pelophylax* complex species (*Pelophylax ridibundus*, *P. lessonae*, *P. esculentus*) with trematodes from family Diplostomidae (*Codoncephalus urniger*, Rudolphi, 1918) was established. For each species there were established: taxonomic status, including synonyms, the hosts, the location in organs, the geographic spreading, etiology and biologic cycle. There are also mentioned the amount of collected material, the morphologic description of the species, original figures and pictures, the level of infestation depending on age, sex and ecosystem.

**Key words:** *Amphibia, infestation, trematodes, family Diplostomidae.*

## **Introduction**

Helminthological researches are frequently focused on study of the infestation level of domestic animals, wild animals, pets and humans. Currently, in different regions of the world most taxa species are poorly studied from helminthological point of view, including amphibians. Amphibians' value as an essential component of biota is quite obvious as the definitive, intermediate, complementary hosts and as host for various groups of helminthes. In some cases, amphibians serve as cause of infections not only for domestic and wild animals, but also are considered as an important agent in maintaining their circulation in nature and actively participate in the formation of parasitic zoonoses (Чихляев, И.В., Кириллов, А.А., Кириллова, Н.Ю., 2012).

That is why, it is appropriate for helminthological study of the amphibians from *Pelophylax esculenta* complex, to establish their circulation specificity in the natural and anthropogenic habitat, but also their contact with the host.

This study will contribute to developing the knowledge base necessary to assess the ecological role of amphibians as definitive, intermediate, complementary, reservoir hosts, as well as biological indicators of aquatic and terrestrial habitats, and will determine the parasitological situation, establishing some features in the pathogenesis of outbreaks of parasitic agents and the development of epizootic and epidemiological measures, increasing knowledge of fauna.

In terms of taxonomic and systematic research, data on faunal and ecological researches in amphibians (fam. Ranidae) can be considered rather complete, but their helminth fauna in the Republic of Moldova for the first time is studied.

## **Material and metodes**

Investigations for determining the level of infestation of amphibians from fam. Ranidae were performed in the laboratory of Parasitology and Helminthology of the Institute of Zoology of the ASM. The amphibian species were identified by external characters (Кузмин, С.Л., 2012).

The amphibians in natural and artificial water basins have been captured during the 2012 - 2016 years, during active life.

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237 of amphibians have been captured, including 165 specimens of *P. ridibundus* species (104 males, 61 females), 44 - *P.lessonae* (35 males, 9 females) and 28 specimens of *P. esculentus* (18 males, 10 females).

Helminth fauna diversity was determined according to standard method proposed by Acad. K.I. Skrjabin, that involves examination all the internal organs of the animal (Скрябин, К. И., 1928)

The collection, fixation, determination and helminthological material processing were performed by the methods proposed by various authors (Сергеев, В.П. и др., 2001, Рыжиков К. М. и др., 1980).

To establish the veracity of data were used the methods of mathematics and statistics.

### **Results and discussions**

The conducted studies denote that amphibians from Ranidae family that inhabit the natural and anthropic ecosystems of the Central Codrii from the Republic of Moldova are widespread. Unlike brown European species (*Rana dalmatina*, *R.temporaria*, *R.arvalis* s.a.), the green ranide, or *P.esculenta* complex, have, preponderantly, a green color body and during the annual life cycle depend largely on aquatic environment, therefore they, usually, inhabiting aquatic habitats and their immediate vicinity.

Due to the fact that water basins in the Republic of Moldova have rich aquatic-terrestrial vegetation, hydrologic and thermal regime, depth, configuration and height of the banks, creates optimal conditions for the whole annual cycle of green ranide life with wide spread both in natural ecosystems, as well as in artificial ones.

The aim of the researches was to establish amphibian infestation from *Pelophylax esculenta* complex with trematodes from *Strigeida* order.

### **Systematic classification:** ([www.faunaeur.org/index.php](http://www.faunaeur.org/index.php))

**Order – STRIGEIDA** La Rue, 1926, Subordo,  
Sudarikov, 1959

**Infraorder – DIPLOSTOMOIDEA**

**Family –DIPLOSTOMIDAE** Railliet, 1919

**Genus – CODONOCEPHALUS** Diesing, 1850

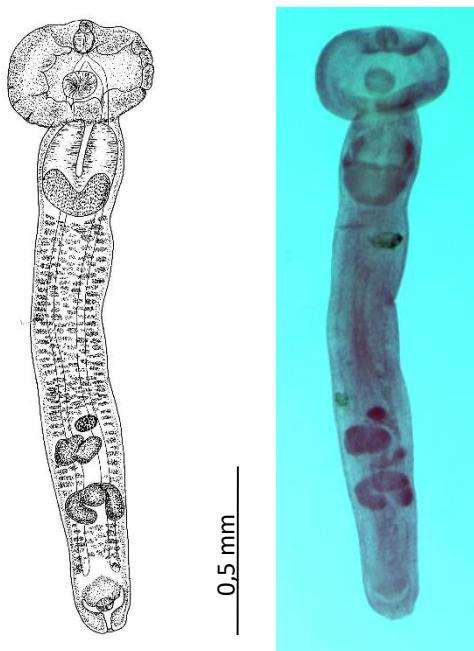
**Species – *Codonocephalus urniger*** Rudolphi, 1819, larvae

**Synonyms** – *Amphistomum urnigerum* Rudolphi, 1819, *Holostomum urnigerum* (Rudolphi) Diesing, 1836, *Codonocephalus mutabilis* Diesing, 1850.

**Hosts** – *Rana ridibunda*, *R. esculenta*, *R.lessonae*, at the occasional infestations also at *Tropidonotus natrix* (Рыжиков К. М. и др., 1980)

**Localization** – Body cavity, subcutaneous cellular-adipose tissue, muscle, various internal organs. At the locating metacercaries in the sexual organs and a high intensity the amphibians' sterility is noted.

**Geographical spread** –European and Asian countries.



**Fig. 1.**  
*Codonocephalus urniger*, larvae - general aspect.  
 Original.

**Foto. 1.**  
*Codonocephalus urniger*, larvae - general aspect.  
 Original.

**Etiology.** The trematode *Codonocephalus urniger* Rudolph, 1819 (Figure 1, Photo. 1) parasites under the skin, into the body cavity, muscles and other organs of the amphibians.

The body length varies from 0.507 to 1.149 mm.

The anterior segment of body has cup-shaped form. The edge opening is wavy or lobed. At the top of the small oval protrusion the cup mouth is located with a diameter of 0.077 to 0.104 mm.

Directly behind the suction cup is the pharynx, whose diameter is 0.127- 0.125 mm. Intestinal thin branches stretch to the rear end of the body. Ventral suction cup with a diameter of 0.192 - 0.208mm, it is located on a flexible stem. The posterior segment is cylindrical and in size - more than the previous one.

Part of the genital atrium is differentiated by a moderate narrowing. The testicles are round, with diameter of 0.286 - 0.369 x 0.239 - 0.358mm.

The seminal vesicle is present. The ovary has dimensions of 0.151 - 0.156mm. Vitelogene glands occupy the space between the previous segments up to genital cone. The uterus is free of eggs. Genital cone is bulky and penetrated by hermaphrodite channels.

**Table 1.** Morphometric parameters of the species *Codonocephalus urniger* Rudolphi, 1819,  
n = 15

Caracters	Mean, mm	MS	$\sigma$	CV	sdCV	Minim, mm	Maxim, mm
Surface	2.851	0.149	0.577	20.2	3.8	1.364	3.447
Body length	5.053	0.208	0.807	16.0	3.0	3.586	5.921
Body width	0.545	0.022	0.085	15.5	2.9	0.337	0.687
Suction cups length	0.077	0.007	0.023	30.1	7.3	0.050	0.117
Suction cups width	0.104	0.005	0.016	15.4	3.5	0.070	0.123
Pharynx length	0.127	0.006	0.0237	18.7	3.7	0.078	0.170
Pharynx width	0.125	0.006	0.021	16.5	3.2	0.094	0.165
Ventral suction cup length	0.192	0.014	0.048	24.8	5.4	0.116	0.279
Ventral section cup width	0.208	0.011	0.037	17.7	3.7	0.154	0.272
First testicle length	0.286	0.029	0.110	38.4	8.3	0.122	0.468
First testicle width	0.369	0.266	0.099	26.9	5.4	0.158	0.568
Second testicle length	0.239	0.019	0.068	28.5	6.0	0.142	0.381
Second testicle width	0.358	0.021	0.076	21.1	4.3	0.208	0.506
Ovary length	0.151	0.012	0.015	29.5	6.0	0.099	0.027
Ovary width	0.156	0.009	0.033	20.9	4.1	0.093	0.215

Note: MS – average error,  $\sigma$  – standard deviation, CV – variation coefficient, sdCV – error of variation coefficient.

At the rear part of genital atrium, around the genital conus the ring crease is well developed. In the posterior segment is a dense network of subcutaneous channels containing fat droplets. Concomitantly with the posterior segment, there are two collecting channels which up to excretory pore are united in one. (*Table 1*).

**Biological cycle.** *Codonocephalus urniger* Rudolphi, 1819 is a trematode with trixen development cycle. As intermediate hosts serve aquatic snails: *Lymnaea stagnalis* and *L. palustris*, amphibians for this trematode are complementary hosts. In their organism the larval form parasitize, at the stage of trematodes metacercaries *Codonocephalus urniger* Rudolphi, 1819.

Definitive hosts are various species of birds as: *Botaurus stellaris* Linnaeus, 1758, *Ixobrychus minutus* Linnaeus, 1766, *Ardea purpurea* Linnaeus, 1766, *Egretta garzetta* Linnaeus, 1766 etc.

Amphibians' infestation takes place beginning with stage of tadpoles and finishing with adult specimens.

According to performed helminthological investigation on the species *Pelophylax esculenta* (*P.ridibundus*, *P.lessonae*, *P. esculentus*) complex in the Republic of Moldova during the 2013 -

2016 years there was established the presence of the species *Codonocephalus urniger* in the body cavity, subcutaneous cellular-adipose tissue, muscles and various internal organs.

At the locating metacercaries in the sexual organs and at the high intensity the sterility of amphibians was established.

Our research performed during the 2013-2016 demonstrates that the level of helminth infestation in *P. esculenta* complex depending on intrinsic and extrinsic factors depends both on the helminth species, as well as the host species.

In all the species of the complex *P. ridibundus*, *P. lessonae* and *P. esculentus* infestation with *C. urniger* was registered only in the summer and only at the specimens of the complex *Pelophylax* collected from natural water basin Ghidighici and basins from the Valea Trandafirilor park.

Helminthological investigations in dependence on the type of host, demonstrated that the level of infestation depends both on the species invasion, as well as hormonal factor of the host. So, the highest level of infestation with *C. urniger* was registered at females of the *Pelophylax* complex, but higher intensity was established in males (Table 2).

**Table 2.** Extensivity and intensivity of invasion in species from *Pelophylax esculenta* complex depending on the host type

Host	Males			Females		
	EI, %	II, ex.		EI, %	II, ex.	
		Min.	Max.		Min.	Max.
<i>Pphelophylax ridibundus</i>	3.2	2	52	5.6	14	33
<i>Pelophylax lessonae</i>	7.1	1	36	10.0	1	9
<i>Pelophylax esculentus</i>	6.4	1	4	9.1	1	1

Another important factor on which depends the helminth fauna diversity in amphibians and also a frequent question in the literature is the age factor. According to our helminthological research there wasn't registered any infestation of juvenile with trematods species *C. urniger*. Thus, the level of helminths infestation in amphibians increases with the host age and dependend on its trophicity.

### Conclusions

1. For the first time in the Republic of Moldova's fauna has been established and described a new species of trematode *Codonocephalus urniger* with medico- veterinary importance.

2. It was established that at the *Pelophylax esculenta* species complex, *Codonocephalus urniger*\_trematodes species has its organic specificity in the body cavity, cellular-adipose subcutaneous tissue, muscles and various internal organs, and at the metacercaries location in the sexual organs with a high intensity the amphibian sterility was established.

3. The level of amphibian infestation with helminthes depending on seasonal factors, depends both on the helminthes species, as well as the host species.

4. Following the helminthological researches of the *Pelophylax esculenta* complex depending on host sex different values were established, so females are characterized by a higher infestation level than males.

5. It has been estimated that the level of amphibians infestation with helminthes captured from natural basins is higher, in comparison with artificial ones, and this divergence occur depending on biotope, on faunistic condition (presence of definitive hosts, intermediate, reservoir) as well as on condition of their environment.

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## Kids goiter: case study

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### Abstract

*Goiter, an iodine deficiency disorder is characterized by enlargement of the thyroid glands. Goiter in neonate occurs when pregnant goats have a low iodine intake or ingest goitrogens. In this paper is noticed an outbreak of goiter in the kids. Kids came from a herd of 30 primitive Anglo Nubian goats, abortion in the last month pregnancy were recorded in 6 goats, 14 goats delivered stillborn kids and six goats delivered 12 live born kids, but 8 of them died within 12 to 72 hours after birth. Clinical, hematological, biochemical and imaging exams were performed at the newborn kids. Following the bacteriological examination for *Salmonella* spp., *Listeria* spp., *Campylobacter* spp., no pathogenic germs were found that cause bacterial abortion in goats. Histopathological examination of thyroid tissue is characterized by hyperplasia of follicular elements as well as of interphilicular connective tissue, intensifying the secretory activity of epithelial cells and accumulation of a considerable amount of colloid. All of the examination confirmed the diagnosis of goiter. To reduce the economical wastage, through therapeutically expenses and animal death rate, we advise all experts, that they must administrate that in the last gestation period supplementation of iodized salt ration and avoiding goitrogens feed.*

**Keywords:** goiter, goat, thyroid, treatment

### Introduction

Iodine deficiency results in the appearance of goiter, which is characteristic of the enlargement of the thyroid gland in all domestic mammals, birds and reptiles (Ricketts M.H. et al., 1985; Falconer, 1987; Rotkiewicz T. et al., 1988; Osame S. and Ichijo S., 1994; Topper M.J. et al., 1994; Schiller C.A. et al., 1995; Kaneko J.J., 1997; Corradini P. et al., 2000; Simon C. et al., 2000; Garner M.M. et al., 2002; Fyfe J.C. et al., 2003). Sometimes, iodine deficiency during pregnancy could be the cause and the kids surviving after birth develop goiter in later stages of life (Vijlder D., 2003). Goiter in utero is caused due to either primary or due to secondary iodine deficiency (Maxi M.G. et al., 2007). Among the incriminated cases in the appearance of kids goiter is the feeding of pregnant females with low iodine feed or goitrogenic compounds that interfere with thyroxinogenesis (brassica plants, soybean byproducts and water with high content of calcium and nitrates) (Blood D.C. & Radostits O.M., 2000; Radostits O.M. et al., 2007; Singh R. and Beigh S.A., 2013; Paulikova I. et al., 2002). A diet rich in calcium will reduce the absorption of iodine in the intestine. Iodine deficiency occurs due to decreased production of thyroxin ( $T_4$ ), stimulation of secretion of thyrotrophic hormone ( $T_3$ ) secreted by the pituitary gland with hyperplasia of the thyroid gland. Clinical signs are more severe in kids compared to lambs, showing a rise in the volume of the thyroid gland, alopecia, weakness (Radostits O.M. et al., 2007; Singh R. and Beigh S.A., 2013).

### Materials and method

In this paper we note an outbreak of goiter in kids. Kids come from a herd of 30 goats primiparous of Anglo Nubian, six goats aborted last month of pregnancy, 14 were born kids dead and 6 calved 12 kids of which 8 died in 12 to 72 hours after birth. The pregnant goats were feed by the owner with goitrogenic plants such as cabbage, but there were no palpable enlargement of the thyroid glands of the dams. Clinical, hematological, biochemical and imaging exams were carried

out in live animals in the Clinics of the Faculty of Veterinary Medicine Iasi. The necropsy, histopathological and bacteriological examinations were performed within the Sanitary Veterinary and Food Safety Laboratory Iasi.

### Results and discussions

A 3-day-old Anglo Nubian female goat was brought to Infectious Disease Clinic of the Iasi Veterinary Medicine Faculty with suspicion of infectious disease. Following the clinical examination, there was the presence in the upper third of the neck of a submandibular bilobata formation that overlays the trachea just below the larynx, symmetrical, united in the distal part (Liklater & Smith, 1993).

At palpation there was a high consistency, lack of local temperature, fluctuation, well delimited with the size of 5.5 x 3.3 x 1.2 cm (Length x width x height) (figure 1). Rectal temperature (39.9° C) and respiratory rate (25/min) were within normal range, heart rate (98 beats/min) was slightly increased. As a result of the clinical examination we suspect goiter.



Fig. 1 - Clinical aspect at goiter on 3 days- old kid

The ultrasound examination (figure 2) was performed and revealed an enlarged, heterogeneous and hypoechogenicity thyroid gland suggested the diagnosis of goiter.



Fig. 2 – Hypoechogenicity thyroid gland (arrows)

The ultrasound examination (figure 2) was performed and revealed an enlarged, heterogeneous and hypoechogenicity thyroid gland suggested the diagnosis of goiter.

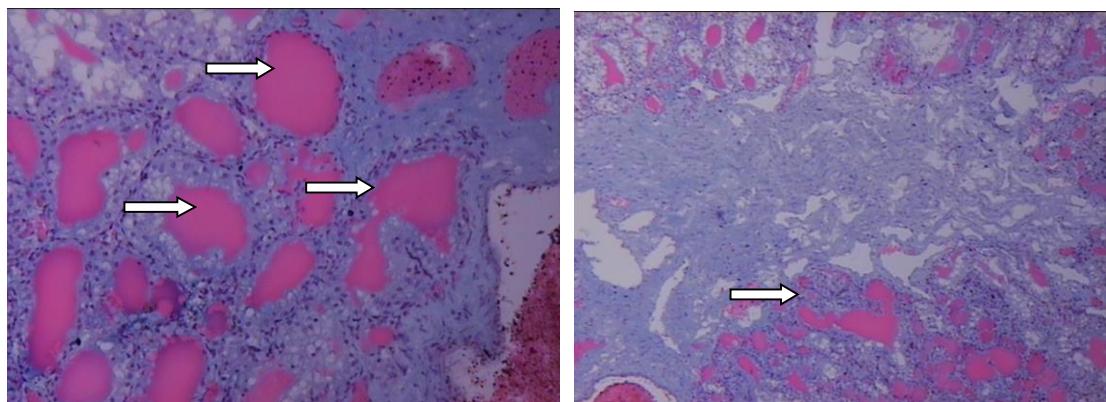
Following the biochemical blood test performed to determine the serum value for thyroid hormones T<sub>3</sub> and T<sub>4</sub>, the following results were obtained: 0.35 nmol/l for T<sub>3</sub> and 47.62 nmol/l for T<sub>4</sub>. Iodine concentration in the blood serum of kids was significant lower in comparison of adequate value such as 4.66+/-2.26 and 182.93+/-2.59 nmol/l, respectively (Bires J. et al., 1996). Low values of T<sub>3</sub> and T<sub>4</sub> are characteristic in hypothyroidism, goiter.

Examination of indicators of the internal environment in the blood serum showed in erythrocyte counts, hemoglobin, hematocrit value, leucocyte counts, revealed a moderate normochromic anemia that is associated with clinical signs of hypothyroidism as well as anisocytosis and polychromasia. Following the hematological examinations we performed, we suspected goiter on kids.

The necropsy exam was performed on an Anglo Nubian male goat weighing 2.4 kg. Removing the skin from the swelling in the neck showed two massive lobes of thyroid gland. These were firm, of moderate consistency and color reddish brown to dark red. The right lobe measured 4.10 x 7.0 cm, while the left lobe was 4.5 cm x 7.6 cm in size. Normally, the thyroid gland accounted for 20% of the body weight of an animal (Kaneko J.J., 1997), in the adult goat it weighs between 5 and 7 grams, the weight was 39 grams of the right lobe and 41 grams of the left lobe. Goiter was diagnosed on the basis of necropsy.

Following the bacteriological examination for *Salmonella spp.*, *Listeria spp.*, *Campylobacter spp.*, no pathogenic germs were found that cause bacterial abortion in goats.

Histopathological examination of thyroid tissue is characterized by hyperplasia of follicular elements as well as of interphilic connective tissue, intensifying the secretory activity of epithelial cells and accumulation of a considerable amount of colloid (figure 3). However, hyperplasia and large quantities of colloid in epithelial cells confirms the diagnosis of goiter in kids (Ozmen O. et al., 2005).



**Fig. 3 - Thyroid follicles with accumulation of colloid (arrows)**

Our clinical and pathological observations indicate those kids has goiter.

The daily requirement for iodine for goats specialized in milk production is 0.8 mg/kgDM (dry matter) and 2 mg/kgDM for rich feed containing goitrogenic plants ([www.bienvivredulaitdechevre.fr](http://www.bienvivredulaitdechevre.fr)), so specialists recommend treating kids goiter aged 4 - 6 months received Levothyroxine sodium, iodized salt and mineral supplements and trace elements (Hassan N. et al., 2013; Vivek J. et al., 2017).

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Because of the too small age, it was not possible to Levothyroxine sodium dosing, so the clinical case of goiter in a 3 days-old kid was successfully treated by local application on the neck skin, to the region of thyroid gland, with aqueous solution of iodine concentration of 100 mg/ml once every two days. The swellings started to regress almost spontaneously and after two weeks of treatment, the size of thyroid was reduced by almost half and iodine applications were made twice a week until the goiter disappeared, that is, four months. It should be noted that iodine can have toxic effects at high levels. It is easily absorbed through the skin especially if repeated over large areas of intact skin or to absorptive mucous membranes (Inchem.org). The kid was fed with healthy animal milk and was administered from the tenth day of life, monthly, per os, 2 ml Selevit (a complex of vitamins and minerals recommended for absorption disorders, growing needs, changes in environmental conditions, changes in feed ration or deficient vitamins and minerals ratios).

## Conclusions

The most common cause of goiter in animals is a deficiency of iodine. Many feedstuffs have goitrogenic effects, and inhibit the activity of the thyroid.

In order to reduce the economical wastage caused both the therapeutic expenses and an increased mortality rate, we recommend supplementing the iodized salt fodder ration as well as avoiding the goitrogenic feeding of goats in the last months of gestation.

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# **Strangulation obstruction of the small intestine in horse**

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## **Abstract**

*Equine colic is a relatively common disorder of the digestive system and should be treated as an emergency. A twist in the small intestine of a horse cause the blood supply to be cut off, resulting in necrotic tissue. The aim of the study was to describe associations between prognostic indicators and diagnoses in equine colic caused by strangulation obstruction of the small intestine. Signs of abdominal pain included: agitation, excessive sweating, flank watching, pawing, frequent lying down, kicking at the abdomen, rolling, tachycardia, absence gut sounds, presence tympanic sounds, abdominal distension, lack of defecation, playing in the water bucket but not drinking and abnormalities at rectal examination. Flunixin meglumine and xylazine were used in every colic case to help control the abdominal pain. Volvulus/torsion of the small intestine resulted in severe pain was difficult to relieve with analgesics. A nasogastric tube was used to relieve the amount of gas pressure in the gut, giving gas and fluids an avenue to travel away from the gut. IV fluids were necessary for dehydrated or in shock horses. Progressive pain with poor response to medical intervention, lack of fever and serosanguineous peritoneal fluid were clinical features that helped distinguish strangulating lesions of the small intestine from simple obstructions and enteritis. Finally horses were euthanized due to grave or poor prognosis. Strangulating obstruction of the small intestine is one of the most lethal forms of equine colic. Minimization of colic episodes depends on management factors, including feeding large quantities of forage, minimizing the amount of concentrate fed and ensuring adequate parasite control.*

**Keywords:** equine colic, small intestine, serosanguineous peritoneal fluid

## **Introduction**

Strangulation obstruction of the small intestine is characterized by simultaneous occlusion of the intestinal lumen and its blood supply inducing rapid deterioration of the intestinal mucosa and subsequent onset of endotoxemia (Blikslager and Marshall, 2015). Any segment of the small intestine may be involved, the distal jejunum and ileum are most frequently affected, most likely because the mesentery is longer in the more distal bowel. Horses have moderate to severe signs of abdominal pain that is only intermittently responsive to analgesic medications (Blikslager, 2010). During the latter stages of the disease process, horses may not experience much pain but rather become profoundly depressed as affected intestine necroses. The prognosis for survival in horses with small intestinal strangulating obstruction is generally lower than with most other types of colic (Blikslager and Marshall, 2015). Treatment includes resection of devitalized bowel, which may not be an option because of the extent of small intestinal involvement. The aim of the study was to describe associations between prognostic indicators and diagnoses in equine colic caused by strangulation obstruction of the small intestine.

## **Materials and methods**

In clinics of our faculty, during the last 2 years (2016-2017) were presented 5 horses mixt breed, 7-14 years old with abdominal distention and severe colic lasting about 2-3 days. They were dewormed with fenbendazole. Previous treatment with antispasmodic and nonsteroidal anti-inflammatory drugs (NSAIDs) made by another veterinarian gave poor response to medical intervention. The diagnosis was made by clinical, hematological, serum biochemistry and peritoneal fluid exams.

## Results and discussions

Horses were presenting signs of severe abdominal pain including: agitation, excessive sweating, flank watching, pawing, frequent lying down, kicking at the abdomen, rolling (Fig 1), absence gut sounds, presence tympanic sounds, abdominal distension, lack of defecation and urination, playing in the water bucket but not drinking, repeatedly going down and getting back up.



**Fig. 1.** Mare, Hungarian Sport Horse, 400kg, 13 years old, rolling



**Fig. 2.** White-grey sticky intestinal mucus in a mare

The most critical examination finding is the horse's heart rate, because this has repeatedly been shown to reflect the level of pain and provide an excellent assessment of the cardiovascular status of the horse. Affected horses have progressive signs of sepsis, including congested mucous membranes, delayed capillary refill time, and an elevated heart rate (60-100 beats/min). Rectal palpation of the abdomen revealed distended by gas loops of small intestine, small amount of feces with thick and sticky mucus (Fig 2). Mucus accumulates on the surface of manure when movement of the intestinal contents slows or stops.

A nasogastric tube was used to relieve the amount of gas pressure in the gut, giving gas and fluids an avenue to travel away from the gut. It was administrated parrafin oil 2 L and water. This increases water content of stool acts as a lubricant for intestinal contents and increase passage of feces.

Peritoneal fluid was typically serosanguinous, with elevations in nucleated cell count ( $>10,000\text{cells}/\mu\text{l}$ ) and total protein ( $>2.5\text{mg/dl}$ ). In acute intestinal obstruction with infarction, the peritoneal fluid is bloodstained (Constable et al., 2017). Abdominocentesis can provide critical information on the integrity of the intestine and is indicated in horses with suspected strangulation of the small intestine (White, 1990). A horse that has signs compatible with a small intestinal obstruction and also has serosanguinous abdominal fluid with an elevated protein level is likely to require surgery, although these cases must be differentiated from proximal enteritis. Horses with small intestinal strangulation show continued signs of abdominal pain, whereas horses with

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proximal enteritis tend to be depressed after initial episodes of mild abdominal pain (Freeman, 2000).

Hematocrit and total protein were increased due to hemoconcentration due to isotonic fluid loss and sequestration into an obstructed or strangulated bowel segment or into the peritoneal cavity. Leukopenia and neutropenia was caused by devitalization of infarcted intestine and the development of endotoxemia. Creatinine, blood urea nitrogen, phosphorus, glucose and lactate serum were increased. Increases in blood (plasma) and/or peritoneal fluid lactate concentrations are useful for prognostication (Constable et al., 2017).

Previous treatment with antispasmodic (butylscopolamine bromide, dose 0.3 mg/kg, IV) and nonsteroidal anti-inflammatory (flunixin meglumine) drugs made by another veterinarian gave poor response. Buscopan® (butylscopolamine bromide) is antispasmodic, antimuscarinic, anticholinergic drug, inhibits secretions and motility of the gastrointestinal tract by blocking parasympathetic receptors. Butylscopolamine bromide is indicated for treating pain associated with spasmodic colic, flatulent colic, and intestinal impactions in horses (Papich, 2011).

Flunixin (1.1 mg/kg, IV) produces analgesic and antiinflammatory effects by inhibiting the synthesis of prostaglandins. It is used primarily for short-term treatment of moderate pain, inflammation and to decrease signs of sepsis. Horses with colic are often treated with low doses of 0.25 mg/kg IV q8h (Papich, 2011). We administrated metamizole (Algocalmin®), doze 10ml, IV. This is one of the strongest non-opioid analgesic drugs, used in both human and veterinary medicine (Baumgartner et al. 2009). At present, metamizole is classified as a non-opioid analgesic (Chaparro et al. 2012, Escobar et al. 2012) although for years it was claimed to belong to non-steroidal anti-inflammatory drugs (López-Munoz et al. 2008, Smith et al. 2008, Dominguez-Ramirez et al. 2010). The most important recommendations declared by manufacturers of veterinary medical preparations containing metamizole are: symptomatic treatment of pain, including colic pain, control of fever in the course of different diseases, meteorism and intestinal constipation in horses, acute and chronic rheumatic diseases, as well as inflammation of the nerves, joints, muscles and tendon sheaths (Jasiecka et al., 2014). Horses received also ketoprofen (Ketink®), 3mg/kg ; 1mL/45kg, IV. Ketoprofen, like other NSAIDs, produces analgesic and anti-inflammatory effects by inhibiting the synthesis of prostaglandins. It has a half-life in most animals of less than 2 hours, but it has a duration of action for up to 24 hours. In horses, ketoprofen is used for musculoskeletal inflammation and pain, abdominal pain, and other inflammatory conditions. In horses, ketoprofen has been less ulcerogenic than phenylbutazone or flunixin meglumine in one study (Papich, 2011).

Administration of xylazine (20mL, IV) which is an alpha2-agonists that decrease release of neurotransmitters from the neuron and is used for short-term sedation, anesthesia, and analgesia in horses, didn't help to control the abdominal pain. IV fluids (NaCl 0.9%, glucose 5%, electrolytes) were necessary for dehydrated or in shock horses.

Progressive pain with poor response to medical intervention, lack of fever and serosanguineous peritoneal fluid were clinical features that helped distinguish strangulating lesions of the small intestine from simple obstructions and enteritis. Gastrointestinal causes of colic that must be differentiated from small intestinal obstructive disease include: gastric ulcer, disorders of the large or small colon, intestinal tympany, thromboembolic colic (Constable et al., 2017).

There is no sign or group of clinical signs which can predict accurately the prognosis for survival of a horse with colic. The best prognosis can be given when referral and medical therapy or surgical intervention occur early in the course of the disease, before the horse's status begins to deteriorate.

The prognosis for survival in horses with small intestinal strangulating lesions is generally lower than for most forms of colic (Blikslager, 2010). The case-fatality rate for horses subjected to surgery is between 30% and 50%, although older reports of the disease had a much higher case-fatality rate (Southwood et al., 2009). Finally one horse was euthanized due to grave or poor prognosis. At necropsy we noticed: large quantity of serosanguinous peritoneal fluid, intestinal serosal surface with numerous petechial and ecchymotic hemorrhages, increased thickness and distended by gas of small intestine loops (Fig 3, 4). The mucosa was deep red and contained petechial hemorrhages and occasional foci of necrosis and ulceration.



**Fig. 3.** Horse. Small intestine distended by gas, thickened loops, serosal surface with petechial and ecchymotic hemorrhages



**Fig. 4.** Horse. Volvulus of small intestine (jejunum, ileum)

Strangulating obstruction may be divided into hemorrhagic and ischemic forms. In hemorrhagic strangulating obstruction, which is most common, the veins become occluded before the arteries because of the greater stiffness of arterial walls. This lesion is noted by a darkened appearance in affected bowel and increased thickness as blood is pumped into the lesion (Blikslager, 2010)

### Conclusions

Strangulating obstruction of the small intestine is one of the most lethal forms of equine colic. A thorough evaluation of the horse with colic allows early identification of cases that need referral for intensive medical or surgical intervention. Early referral improves the horse prognosis and reduce client costs by allowing intervention while the horse is systemically stable. Minimization of colic episodes depends on management factors, including feeding large quantities of forage, minimizing the amount of concentrate fed and ensuring adequate parasite control.

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# **Detection of BHV-1 antibodies in bovine raised in rural households from Iași County**

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## **Abstract**

*Infectious bovine rhinotracheitis / infectious pustular vulvovaginitis (IBR/IPV) is a significant disease among domestic and wild cattle. IBR/IPV is caused by Bovine Herpesvirus-1 (BHV-1) that is capable of attacking many different tissues in the body leading to a variety of clinical diseases. The virus can persist in clinically recovered animals for years, remaining inactive until the animal is placed under stress. Investigations were undertaken during 2015-2016 on bovine raised in rural households in Miroslăvești and Ciohorăni commune from Iași County. Individual serum samples from 305 cattle were tested for detection of BHV-1 antibodies using an ELISA commercial kit (IDEXX IBR Ab). The results showed that overall seroprevalence of infectious bovine rhinotracheitis (IBR) was 63.6% in tested cattle. These data underline the fact that in household reared cattle are several animals with BHV-1 lifelong latent infection.*

**Key words:** BHV-1, bovine, antibodies

## **Introduction**

Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV) is a highly contagious and infectious viral disease that affects cattle of all ages. The disease is characterized by inflammation of the upper respiratory tract. The virus that causes IBR, bovine herpes virus 1 (BHV-1) also causes infectious pustular vulvovaginitis in the female, and infectious balanoposthitis in the male and can cause abortions and foetal deformities (Jones C, Chowdhury S., 2007).

Infected cattle develop a latent infection once recovered from the initial infection and despite appearing clinically normal may suffer recrudescence of disease when under stress. Exposure to BHV-1 may be highlighted by antibody detection. In most cases, cattle are able to overcome a primary BHV1 infection, so the primary immune response provides valuable information for primary, secondary, and passive immunity. The main immune response to the virus and virus-infected cells is to the viral envelope glycoproteins gB, gC, and gD (Engels and Ackermann, 1996).

## **Materials and methods**

Investigations were undertaken during 2015-2016 on bovines raised in rural households in Miroslăvești and Ciohorăni commune from Iași County. Individual serum samples from 305 cattle were tested for detection of BHV-1. All bovine submitted to test were not prior vaccinated against any viral respiratory disease. Prior to analysis all serum samples were stored at -20°C.



**Fig.1** Miroslăvești Commune  
(Mitești, Verșeni, Soci, Miroslăvești vilages)



**Fig. 2** Ciohorăni Commune

A commercially available screening blocking ELISA kit was procured from HerdChek, Idexx Laboratories. The kit was used strictly in accordance with the manufacturer's instructions.

### Results and discussions

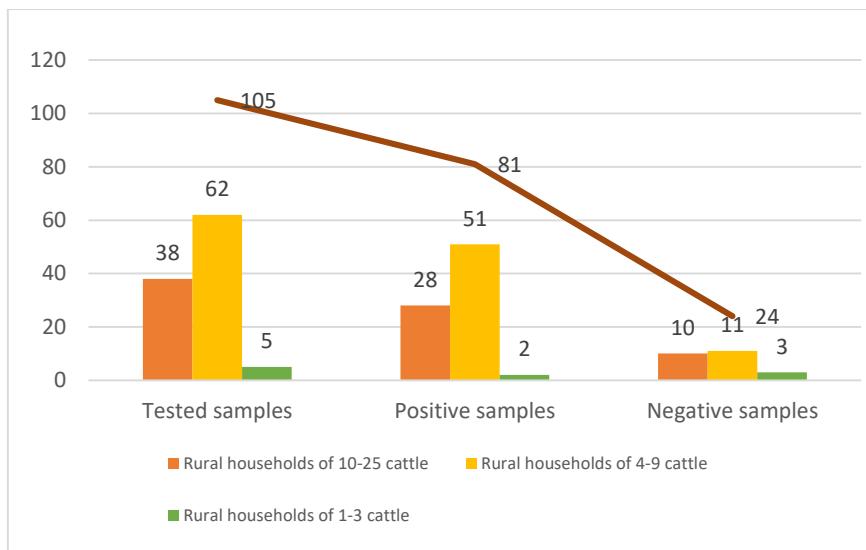
Specific antibodies to BHV-1 can be first detected at 7 to 10 days post-infection. After the acute phase and during latency, BHV-1-infected cattle are mainly detected by the presence of specific antibodies to BHV-1 (Jones C. et al, 2006). Enzyme-linked immunosorbent assay (ELISA) is a sensitive and specific test in terms of detection of the low level of antibody for several viral diseases; this has been extensively used in recent years by many researchers to highlight the seroprevalence of IBR in cattle population (Muylkens B et al., 2007).

Out of 305 serum samples screened, 194 (63.60%) were found to be positive AB-ELISA for BHV-1 antibodies (Table 1). At period level seroprevalence of IBR antibodies was highest in 2015 (77.14%) and lowest in 2016 (56.50%).

**Table 1.** Serologic results of BHV-1 antibodies detection in bovine raised in rural households from Iași County

Year	Samples tested	Positive samples		Negative samples	
		No.	%	No.	%
<b>2015</b>	105	81	77.14	24	22.86
<b>2016</b>	200	113	56,5	87	43,5
<b>Total</b>	305	194	63,6	111	36,4

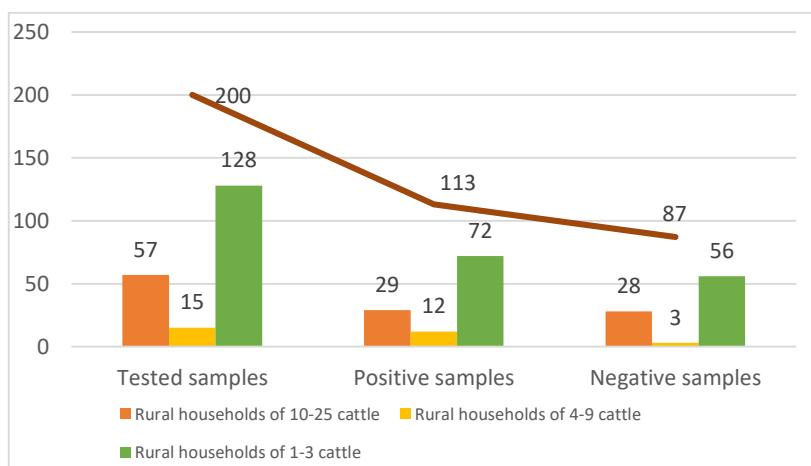
Investigations undertaken during 2015 revealed a higher seroprevalence in rural households of 4-9 cattle (82%) compared to rural households of 4-9 cattle (73%).



**Fig. 3** Serologic results of BHV-1 antibodies detection in bovine in 2015

Our results indicate that BHV-1 is widely disseminated in cattle population raised in households from Iași County. The variation in seroprevalence rate among different size households can be attributed to management in all aspects, especially introducing of purchased animals.

The overall seroprevalence obtained in 2016 (56.50%) was lower than the one in 2015. Prevalence rate among different size households had the same pattern as in 2015, with higher percentage for rural households organized in 4-9 cattle. Consequently farmers need to be aware that they must increase their efforts to avoid the introduction and spread of BHV-1 to their herds, to establish the health status of their herds and determine what mitigation measures are required to address.



**Fig. 4** Serologic results of BHV-1 antibodies detection in bovine in 2016

BHV1 infection is commonly diagnosed serologically. The presence of the BHV-1 infection has been demonstrated through studies conducted in previous years on cattle in eastern

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Romanian counties (Aniță D. et al, 2013; 2010). Identifying seropositivity following natural BHV-1 infection in cattle population means that some of the animals in that herds may have a latent infection and that these animals should, from an epidemiological perspective, be viewed as carriers and transmitters of the virus (Tuncer P, Yeşilbağ K., 2015). Serological testing and removal of infected animals has been successfully used to eliminate BHV-1 from Denmark, Switzerland and Austria (Ackermann and Engels, 2006).

### **Conclusions**

Overall seropositivity rate of BHV-1 antibodies in bovine raised in households from Iasi County was 63.60%. Cattle identified as seropositive have an increased risk of virus transmission and recurrent disease. Detection of anti-BHV-1 antibodies in bovine raised in households reveals the need to implement control measures regarding the Infectious Bovine Rhinotracheitis (IBR).

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# Molecular detection of hepatitis E virus in wild boars from Botoșani County

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## Abstract

Hepatitis E virus (HEV) infections occur in both humans and animals worldwide. The domestic swine and wild boars (*Sus scrofa*) are known as important reservoirs of HEV, zoonotic hepatitis E infections of foodborne origin being linked to consumption of pig, wild boar and deer raw or undercooked meat or undercooked products. The aim of the study was to highlight the presence of HEV in wild boars using molecular biology methods. During hunting season 2015-2016 were collected blood and faecal samples from 22 wild boars from Suharău hunting fund in Botoșani County. Using degenerate primers, HEV RNA was detected by nested reverse transcriptase PCR in all wild boar faecal samples. The blood (EDTA whole blood) samples tested were negative for hepatitis E virus RNA. Our results indicate that wild boars are a possible source of infection for pigs and human population in Botoșani County.

**Keywords:** HEV, wild boar, RNA

## Introduction

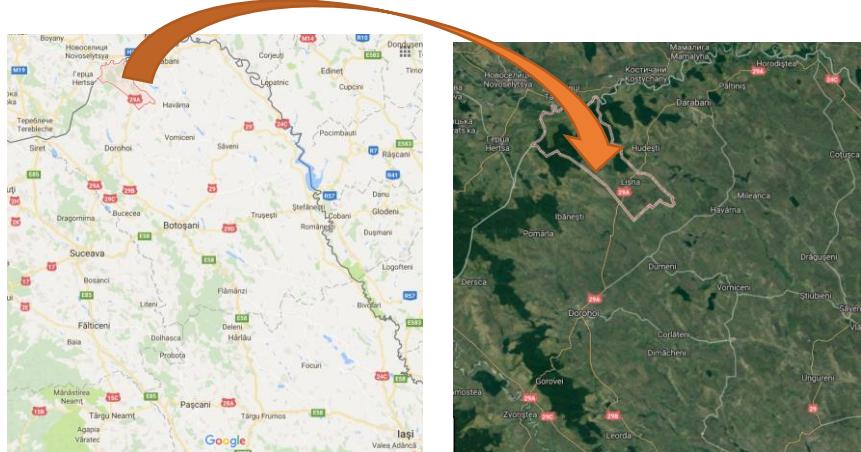
Hepatitis E virus (HEV) is a viral pathogen transmitted primarily via fecal-oral route, classified in the genus *Orthohepevirus*, the family *Hepeviridae* (Smith et al., 2014). Human pathogenic HEVs are mainly classified into genotypes 1–4. Although genotypes 1 and 2 infect only humans, genotypes 3 and 4 are zoonotic and infect different animal species and humans. The main animal reservoirs for genotype 3 are domestic pigs and wild boars (Pavio N. et al., 2015).

The zoonotic transmission of hepatitis E virus (HEV) is of special concern. Direct zoonotic transmission to humans has been documented several times from wild boar and pigs infected with HEV. High HEV seroprevalences can be found in European pig and wild boar populations (Van der Poel WH. 2014). To determine wild boar hepatitis E virus (HEV) we analysed molecular markers of HEV infection among feral pigs in Botoșani County.

## Materials and methods

Investigations were undertaken during hunting season 2015-2016 on wild boar samples from Suharău hunting fund in Botoșani County. Ten faecal samples and 12 EDTA whole blood samples were collected from 22 animals. Prior to analysis all samples were stored at -80°C.

HEV detection protocol consisted in the following steps: RNA extraction, reverse-transcription and nested PCR using specific HEV primers. RNA extraction was achieved using two extraction kits: QIAamp Viral RNA (Qiagen) for faecal samples and RNAeasy Mini kit (Qiagen) for EDTA whole blood samples according to the manufacturer's instructions.



**Fig. 1.** Sampling area: Suharău hunting fund in Botoșani County

Detection of HEV RNA from faecal and EDTA whole blood samples was performed by nested RT-PCR targeting a fragment of the structural gene of HEV in ORF2 using primers 3156N and 3157N (730 nt product) for the first PCR and primers 3158 N and 3159 N (348 nt product) for the second PCR (nested-PCR) (Table 1). PCR products were separated on a 1% agarose gel and visualized using Gel Doc Documentation System (BioRad).

**Table 1.** Sets of primers used for nested rt-PCR for HEV RNA detection

Primer	Target gene	PCR method	Sequence	Location	Polarity
3156N	ORF2	PCR	AATTATGCC(T)CAGTAC(T)CGG(A)GTTG	5687–5708	Positive
3157N	ORF2	PCR	CCCTTA(G)TCC(T)TGCTGA(C)GCATTCTC	6395–6417	Negative
3158N	ORF2	nested-PCR	GTT(A)ATGCTT(C)TGCATA(T)CATGGCT	5972–5993	Positive
3159N	ORF2	nested-PCR	AGCCGACGAAATCAATTCTGTC	6298–6319	Negative

## Results and discussions

Our previous investigations on hepatitis E in susceptible species from Botoșani County highlighted the existence of a reservoir of infection. Previous results revealed the presence of hepatitis E infection in human population and pigs reared in household system (overall prevalence - 22.66%) in Botoșani County (Aniță A. et al, 2010).

In most cases of autochthonous hepatitis E in developed countries the source and route of infection cannot be identified. However, the evidence suggests that most cases may be due to consumption of undercooked HEV contaminated pork or game meat (Nan Y. et al, 2017). The dynamic of HEV infection in wildlife is still unknown, but recent research highlights the maintenance and circulation of the virus, posing a risk to human health in the case of meat consumption from wild boar and deer (Anheyer-Behmenburg, H.E. et al, 2017).

The aim of this investigation was the detection of molecular markers of hepatitis E infection in wild boars from Botoșani County. Samples (faeces and EDTA whole blood) from 22 animals were tested for the presence of HEV. Using nested RT-PCR the presence of hepatitis E

virus was detected in all faecal samples. Wild boar EDTA whole blood samples were negative for HEV genome (Fig.2).



**Fig. 2** Migration of the PCR products after nested PCR amplification  
1F, 2F, 3F – wild boar faecal samples  
1S, 2S, 3S, 4S, 5S – EDTA whole blood samples

HEV has been frequently isolated from wild boars in Europe and in Asia. For the first time, it was detected in wild boars in Japan, and the genotype was the same as most frequently seen in production pigs, HEV-3 (Sonoda H. et al, 2004). Since then, isolation of HEV RNA from wild boars has shown that these animals are important hosts of HEV also in several European countries: Spain (Martelli et al., 2015), Germany (Vina-Rodriguez et al., 2015), Sweden (Roth et al., 2016), Italy (Oliveira-Filho et al., 2014; Di Profio et al., 2016), Slovenia (Zele et al., 2016) and Portugal (Mesquita et al., 2014). Hepatitis E virus was first detected in Romania 2017 by Porea D. et al, highlighting the potential role of wild boars as zoonotic reservoir for HEV.

### Conclusions

The results of our investigations revealed the presence of HEV infection in wild boars from Botoșani County. Since large amounts of virus particles are excreted in feces of wild boar, droppings can contaminate the environment and pose a particular risk to susceptible species. Zoonotic hepatitis E infections of foodborne origin have been linked to consumption of pig and wild boar meat, mostly uncooked or undercooked products, raising public health concerns.

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# **West Nile virus infections in Romania after the 2010 outbreak- a retrospective study in human and animal population**

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## **Abstract**

*In this study the authors have made a retrospective analysis of West Nile virus infection in human and animals after 2010. Few studies have dealt with this arbovirosis in the last years and there is no analysis to correlate the evolution in animals and humans. To date it is considered that the largest outbreak of West Nile virus (WNV) encephalitis from Europe was registered in 1996 in Romania. Cases of encephalitis with WNV have been reported in our country before first in 1955 in Transylvania, followed by an outbreak in 1964 in Banat. The outbreak from 1996, ended with over 390 confirmed cases of encephalitis in humans the majority being registered in Bucharest and in the southern area of Romania. The epidemiological situation, after the implementation of a surveillance system for WNV in humans until 2010, was characterized by sporadic cases, WNV being reported yearly, except 2002. The infections were reported mostly in the south and south eastern area of the country. The age distribution in human cases recorded a majority in the group age over 65 years due to the senescence of the immune system and the associated diseases. The gender ratio was dominant for men, considering the occupational factor. In 2010 the second large outbreak of WNV was recorded and 47 confirmed cases of WNV infection were registered. After 2010, WNV encephalitis was recorded yearly and the area of detection was confined to eastern area and slowly to the whole country. Human cases were registered yearly in areas where the virus was detected in animals by serological screening. Considering the analyzed data, we can state that the main feature of WNV infection is the continuous virus spreading over the territory both in humans and animals. This can be determined by the climatic changes, that allow the surviving and multiplication of the competent vectors in new areas and maybe of the changes in the migratory routes of the birds- main amplifying hosts. Unfortunately, no statistical correlation between human and animal cases could be made. We can state that WNV is continuing to represent a threat for public health and more in depth research has to be made in order to characterize the evolution of the infection in our country and the causes of its endemisation.*

**Key words:** West Nile virus, distribution, human, animal

## **Introduction**

Vector borne diseases represent a major threat and concern to human health all over the world. By far, the most widespread and current infection with an arbovirus, is the infection with West Nile Virus (WNV), which was reported in Romania even before the biggest outbreak from our country (1996), such being reported since 1955 in Transylvania and in 1964 in Banat county. These confirmed outbreaks were followed at an erratic interval (1996) by a sudden recrudescence, developed in the biggest outbreak of WNV infection in Europe, in urban settings, to date, with a high rate of neurological infections. In 1996, between July 15 and October 12, there were identified 393 serologically confirmed or probable patients with WNV infections. Out of them, 352 had meningitis. Affected patients were distributed among 19 districts in the southern, western, central and eastern parts of the country (Sirbu A., et al., 2011). Regarding age, fatality and incidence increase with age, hence, 17 patients older than 50 years died (Tsai TF et al., 1998).

Until the second major outbreak, between 1997 and 2009, the epidemiological status of the country, was characterized by sporadic cases reported on the south side of Romania (Campbell GL., et al 2001), and according to National Institute for Public Health, the confirmed cases with

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WNV were reported yearly, except 2002. Also in this year, the surveillance system set in 1997 was extended to a national level (Sirbu A et al., 2011).

In 2010, the epidemiological phenomenon begun on May and ended on November 15<sup>th</sup>, and materialized with a total of 170 suspected cases with WNV infection reported at the national level. Of these, 52 have been confirmed by laboratory diagnosis, 5 were probable cases, while the rest of them were clearly negative for WNV (Sirbu et al. 2011). Among the confirmed cases have been reported four deaths (two deaths in Constanta county, one in Bucharest and the other one, in Mehedinti county) (ECDC, 2010). The distribution per age, show that the median age was 53.4 years (age range: 12-81 years) (Sirbu et al., 2011), while all the deaths were reported in person over 65 years old (ECDC, 2010).

In the 2010 outbreak, the highest incidence was reported in Ialomita county and Constanta county, which are located in the south-eastern part of the country, in the Danube Delta, where are reported most of WNV infection (ECDC, 2010).

If the human cases are reported yearly, the seroconversion in animals is detected only due to serosurveillance assessments, while most of the researching focus on the detection of antibodies or virus in horses, known as dead-end hosts, and birds, who act as amplifying hosts and also as a reservoirs. Once with the human epidemic from 2010 the OIE.int, reported two outbreaks with WNV in horses (five cases) in Braila County and one outbreak (one case) in Constanta County, without any correlation with human cases from that time (OIE.int, 2010).

### **Synopsis on chronological evolution on WNV infections in Romania**

After the second major outbreak, in Romania, the infection with WNV in humans was reported yearly, the dynamic marking an increasing trend until 2016, while in the next year, the human cases have recorded in lower values (CNSCTB, 2011, 2012, 2013, 2014, 2015) (ECDC, 2016, 2017).

In 2011, the National Institute of Public Health reported 11 human cases (ten confirmed and one probable), most of them in Bucharest (five confirmed cases). Additionally, in the same year, two cases were reported in Constanta, one in Galati, both of which are known to be at risk of transmitting WNV. Also, there have been confirmed cases in Iasi and Tulcea. Since the beginning of the season, most cases have been observed in August (six cases), while the others were in September (three cases) and July (two cases).

Age distribution shows that elderly people are more likely to develop WNV infections due to immunological senescence and also due to other conditions affecting the body's defense mechanism, diabetes and cardiovascular disease (Kleinschmidt-DeMasters, BK et al., 2004), while a 2001 study after the outbreak in New York (1999) shows that the incidence of neurological signs is ten times higher in people aged 50 to 59 and 43 times higher in older people aged 80 (Nash D. și col.,2001).

For the same year, the seroconversion induced by WNV in animals was detected in serum samples collected from migratory and resident birds, from Buzau, Braila and Tulcea during their breeding season. The results show that out of 53 serum samples, 20 had antibodies anti-WNV Ig G-type (Pastiu AI et al., 2016).

Presence of specific antibodies anti WNV, were detected in horses from six counties from the eastern side of Romania (Bacau, Braila, Galati, Iasi, Vrancea and Tulcea). In this study, a total of 808 serum samples were collected and tested, and out of all, 473 were Ig G positive (Ludu et al., 2014).

The results obtained from serological screening in animals in seven counties in the eastern part of Romania complete the classic virus transfer route, involving transmission from invertebrates

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to vertebrate hosts, that could act as amplifying hosts, (birds) and horses that can not transmit the virus due to low levels of viraemia, and also confirm that horses remain a good indicator of WNV presence (Ludu et al., 2014).

In 2012, the evolution of WNV infections in humans, was marked by a slight increase in cases. According to the National Institute of Public Health, in 2012, 14 suspected cases were reported. Of these, 13 have been confirmed by laboratory diagnosis. The most cases were recorded in August (11 cases), while the rest of them were in September (two cases) and July (one case). The spatial distribution of cases encompasses counties from the south side of Romania (Ialomita, Ilfov, Giurgiu, Braila and Bucharest), but also Iasi county.

The gender ratio between men and women was 1.1, the ratio would be assumed by incriminating the occupational factor. As it was in the previous year, the most affected were the elderly, in 2012 being reported the death of a 87 years old woman.

In animal's populations, the presence of specific antibodies anti-WNV were detected in birds and horses. According to EFSA, the sampling strategies involved the collection of serum from horses in three villages from Constanta and Braila, areas where the Ig M conversion were found in previous year. In June, August and October were collected 45 serum samples from Constanta County and 183 from Braila County. The detection of Ig M was positive in only one sample (from Braila County) out of 228 samples and the rest of them negative. Finding one positive case meant that the viral circulation was still present in that region.

The detection of anti-WNV antibodies in birds (migratory and resident) in the same study conducted by Pastiu AI et al, also confirm that the virus circulates among birds from the Danube Delta region. This area represent an important region of passing, feeding, nesting for migratory birds, and for epidemiological importance, this area might be an important way of introducing the WNV by migratory birds (Prioteasa F.L et al., 2007). In this year, 31 serum samples were collected from the same region, and the seroconversion was positive in seven of these.

In 2013, the epidemiological situation shows a slight increase in human cases with a wider distribution, while the detection of seroconversion in animals proves that WNV persists among the horses in the areas tested in previous years.

Within the national surveillance study implemented after the 1996 epidemic, in 2013 have been reported 24 cases and of these, 22 were positive. As in previous years, the most affected people were the elderly, and according to National Institute of Public Health, there was no death reported. Since the beginning of season, most of cases were registered in August (17 cases). In September were six cases and in July, only one reported case. The annual report didn't mention whether patients reported travels in endemic areas, mosquito bites, stagnant water around the dwelling, gardening or poultry raising, although these are potentially risk factors that contribute to upstream epidemiological research.

Eleven counties (out of 42) reported confirmed WNV infection in 2013. The highest incidence was reported from Braila county (four confirmed cases), Ialomita county (three confirmed cases) and Tulcea county (two confirmed out of three reported). Confirmed cases were also reported in Bacau, Bucharest, Constanta, Galati, Iasi, Ilfov, Mures, and Sibiu counties. Confirmed cases in the Mureş and Sibiu counties were reported for the first time and the spatial distribution for the rest of the reported cases (the eastern and southern parts of Romania) confirm the viral circulation along the Danube Delta and its persistence in the areas where most of the WNV infections have been reported over time.

If the epidemiological and virological situation of WNV infection in humans was marked by the increase in confirmed cases, the spread of the virus in new areas (Mures and Sibiu counties)

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and its persistence in endemic areas (Constanta, Tulcea, Bucuresti counties), the assessment of the national situation, involved the sampling from areas where Ig M conversion were found in 2011.

Serological surveillance in animals comprised areas from three villages in Constanta (56 serum samples) and Braila counties (25 serum samples). The results obtained by testing serum samples collected from domestic horses in June, August and October show that there was no viral circulation in these areas during the 2013 season (European Food Safety Authority, *Trends And Sources Of Zoonoses And Zoonotic Agents In Humans, Foodstuffs, Animals And Feeding Stuffs*, 2013).

A study by Ludu L. and others in 2013, which included the collection of 110 samples of horse serum from Buzau (ten samples), Braila (40 samples) and Tulcea counties (60 samples), proves the opposite of the results obtained by the monitoring EFSA. The sampled horses didn't have any history regarding epidemiological movement.

Of the 110 samples, 60 were randomized and tested for anti-WNV antibodies (IgG type). Out of the 60 serum samples tested for the presence of anti-WNV IgG, 32 were positive. Seroprevalence in the target areas was 53.3%. IgG was confirmed in 24 of the 32 samples in Tulcea county, in 8 out of 20 samples collected from Braila County and in no evidence from those collected in Buzău (0/8). For the detection of Ig M, from all the positive samples (32), only two were positive for Ig M: one from Tulcea county and the other one from Braila. Considering the seropositivity, it is assumed that the virus circulated in those areas.

The difference in seropositivity obtained in the two studies (EFSA, no positive sample, Ludu et al., two IgM-positive samples, both collected from Braila county) can be attributed to the fact that IgM may be present between three and eight days after the onset of the disease and may persist for up to 30 -90 days, whereas IgG may persist many years (Terrestrial Manual, OIE.int, 2013).

Although mosquitoes, primarily of the genus *Culex*, are the main vectors (Hubálek Z, et al., 1999) both soft and hard ticks can be infected with WNV, but are unlikely to play a significant role in transmitting the virus (Hayes EB, et al., 2005). However, WNV has been isolated from ixodid and argasid ticks in Europe, placing them secondly after mosquitoes, as well as their importance as vectors (Lawrie CH, Et al. 2004) and their potential to act as a reservoir of the virus (Lawrie CH., et al. 2004). In the same year, in Romania, it was shown that ticks are involved in natural transmission, they can act as a replacement vector, resulting in a bird-tick-bird cycle (Koustukov MA, et al., 1985). The WNV was detected in an immature tick (nymph), identified as *Hyalomma marginatum marginatum*, collected in Danube Delta Biosphere Reserve, at the end of August 2013, the finding emphasizing the role of ticks in introduction and maintenance of the virus in infections (Kolodziejek J., et al., 2014).

In 2014, within the national surveillance system, have been totally reported 24 human cases, after the onset of the season. Most of the cases were reported in August (14 cases), as it was in previous year, while in September were reported eight cases and in October, two. Cases, though not many, covered the south and southeast side of country, known as being endemic (Ialomița, Giurgiu, Braila, Constanța, Galati, Valcea, Olt, Dolj, Teleorman, Dambovița, Prahova and Bucharest counties), the central (Sibiu and Mures counties) and the northeast (Iasi county) sides of the country, the occurrence of cases in newly areas, showing a wide viral circulation.

In terms of demographics, especially gender ratio and distribution by age groups, in 2014, both the age and the sex ratio, was as in previous years. The average annual incidence of WNV infections, is higher with increasing age (the most affected age was above 65 years old), and the distribution by gender, was higher among males than among females. The associations between increasing age and a higher incidence of WNV infections among males are not clearly, but it might

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be related to either reporting biases or the presence of underlying conditions that might be risk factors for the development of the disease (Lindsey NP., et al.2010), due to the occupational factor related to open-air occupations.

The serological investigation in animals shows a continuity of the sample collection of sera from horses from Constanta and Braila counties and the sampling of sera from backyard domestic birds, during a serological evaluation that took place 2011 - 2014.

The strategy involved sampling from 45 Braila County and 161 from Constanta County, and all of them (206 serum samples) were negative for Ig M (EFSA, 2014).

Regarding the serological screening for birds, 21 serum samples were collected from private households in Danube Delta. The samples were from *Gallus gallus domesticus* (n=16) and *Anas platyrhynchos domesticus* (n=5), and tested for detection of Ig G-type antibodies anti-WNV. As a result of testing, seroconversion induced by WNV was observed in both *Anas platyrhynchos domesticus* and *Gallus gallus domesticus* (Pastiu A.I., et al, 2016).

In 2015, there was an increase in diversity in the species studied. Thus, in addition to the collected samples from the horses, during the monitoring program, samples from wild boars were also collected in order to demonstrate the seroconversion and the overcoming of the species barrier of the virus.

The samples from horses (n=171) were collected from the same areas that were targeted in previous years, Braila (n=129) and Constanta county (n=42), and the aim was to demonstrate the viral circulation or its persistence in those areas. Out of these, 12 samples from Braila county were positive for antibodies anti-WNV (Ig M), showing a persistence of virus circulation in this region (EFSA, 2015).

The purpose of this study was the detection of specific antibodies in other species but birds and horses, and to prove that there are many susceptible species to infection, both domestic and wild, more accurate, the author's aim was to assess the seroprevalence of the specific anti WNV Ig G antibodies in wild boars. For this purpose, there were collected serum samples from Bacau (n=30) and Galati (n=38) counties. Out of these, 16 samples from Bacau county and 27 samples from Galati county were positive. These results, prove the circulation of WNV or/and other related flaviviruses among wild boars (Paslaru A., et al, 2015).

In humans, since the beginning of the season, the incidence rate has been 32 cases. Most cases occurred in September (15 cases) and August (13 cases), the rest of cases being recorded in July, October and May. Demographic aspects such as, age distribution, shows that the most affected were elderly, and the gender distribution showed that majority of WNV infections were in male.

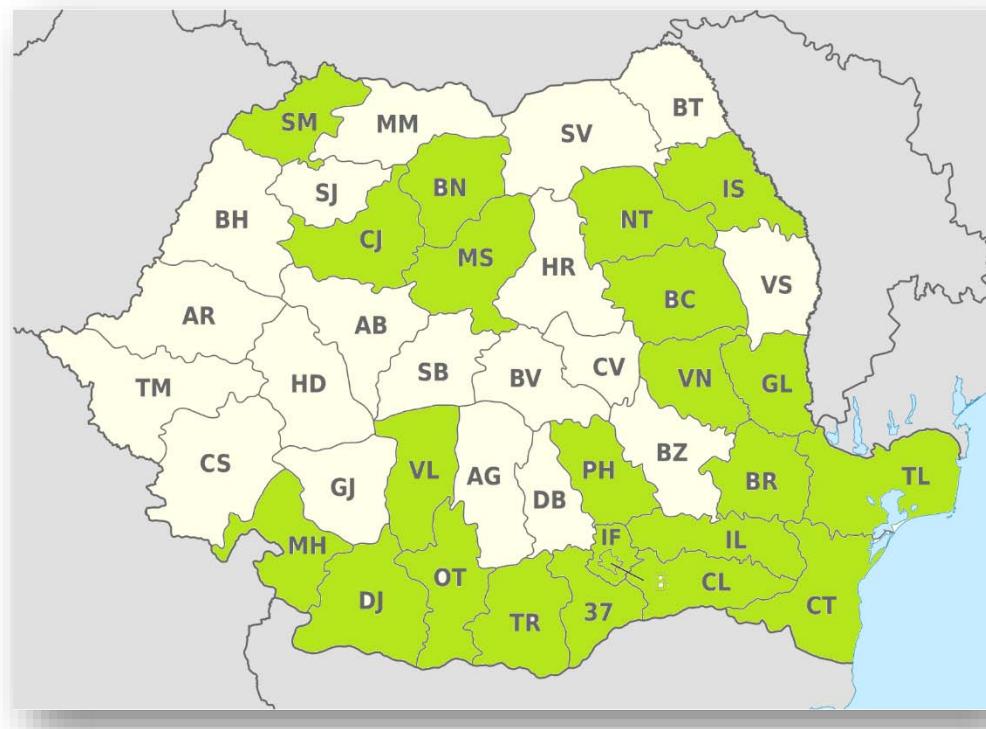
The cases have been recorded in the south and southeast sides of Romania (Argeș, Buzău, Brăila, Ialomița, Giurgiu, Constanța, Dolj, Mehedinți, Ilfov and Bucharest Counties), in central and west side of the country (Sibiu, Cluj and Bihor counties) and in the northside (Iasi County) (CNSCTB, 2015).

2016 recorded the highest incidence, since the 2010 outbreak. If in previous years the incidence's rate had a constant value, in 2016, the incidence value was almost three times higher (n=93) than the one recorded in 2015 (n=32), the year with the highest number of cases between 2011 and 2015.

Out of the total number cases with WNV infection, 85 were classified as confirmed, and the rest of them were probable. The cases were recorded from July 1<sup>st</sup> to October 10<sup>th</sup>, and the peak of incidence was in August and September.

The spatial distribution of human cases, shows a continuation of the virus's existence in this area, and also confirms its existence in the south and south-east of the country, known as

endemic area. Cases were also recorded in the north side of the country and for the first time, WNV was detected in Satu Mare County (Figure 1).



**Fig. 1** Spatial distribution of human cases in 2016

The most of cases were recorded in Bucharest (n=18) and in Braila county (n=17). In Bucharest, besides the increased number of confirmed cases, there were registered also the most deaths (n=6), and although the Mures and Teleorman Counties recorded only one cases, in both cases, the patients died (Table 1).

**Table 1** The incidence of WNV infections in humans in 2016  
Confirmed

County	cases	Deaths
Bacau	7	2
Bistrita	1	0
<b>Bucuresti</b>	<b>18</b>	<b>6</b>
Braila	17	1
Calarasi	1	0
Constanta	3	0
Cluj	1	0
Dolj	3	1

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Galati	8	0
Giurgiu	4	2
Ialomita	5	2
Iasi	5	1
Ilfov	6	1
Mehedinti	1	0
<b>Mures</b>	<b>1</b>	<b>1</b>
Neamt	1	0
Olt	3	0
Prahova	3	1
Satu Mare	1	0
<b>Teleorman</b>	<b>1</b>	<b>1</b>
Tulcea	2	0
Valcea	1	0
Vrancea	3	0
Total	93	19

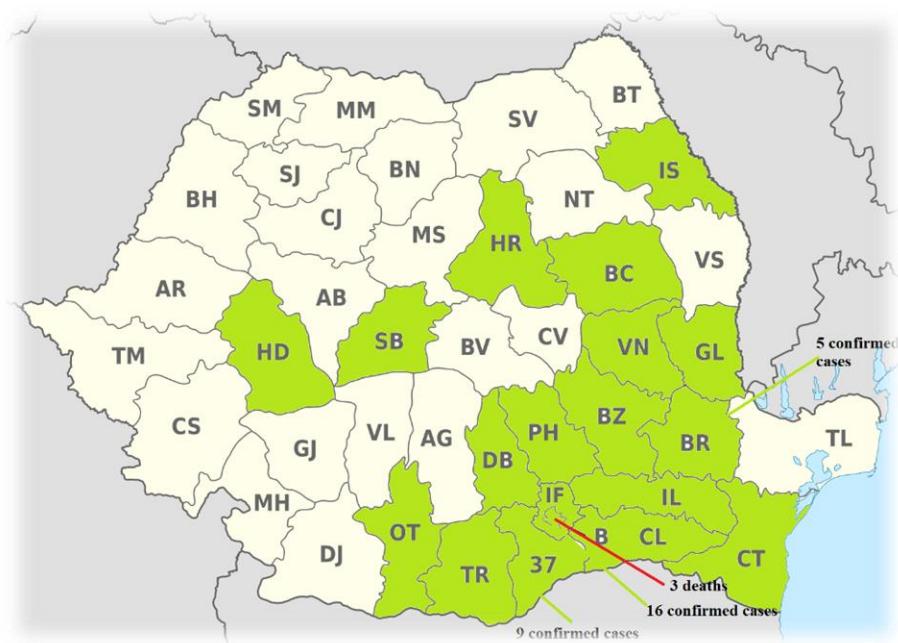
Regarding the demographic characteristics, the average age was 61.9 years (maximum 88 years and minimum 15 years). Out of all cases, 51 of them were female patients and the rest of them, were male. According to National Institute for Public Health, there are no differences between sex distributions, this is an exception to the fact that men would be more prone to WNV infections.

All the confirmed cases admit the mosquito's bites outdoors and none of them received blood transfusion or an organs transplant. The risk factors for all the deaths were represented by premorbid conditions like heart diseases, alcohol abuse, chronic renal disease, neurological affections, diabetes, hepatitis B virus and malignancies. However, according to a 2005 study, diabetes, represent a risk factor for developing neuroinvasive disease (Cynthia M.J et. al., 2007), also it is a predictor of severe illness (Patnaik JL et al., 2006). Diabetic patients are more prone to WNV infection, due to diabetes' role in impairing immune status may lead to an increase in the magnitude and duration of viremia (Campell GL., et al., 2002). Out of all the risk factors, alcohol abuse, diabetes and hypertension, were more commonly noted in the medical records in a descriptive study conducted in 2003 (Bode AV., et al., 2006)

In 2016, the antibodies against WNV have been detected in dogs from four counties from the east side of Romania, within a study that involved the collection of samples from areas where in previous years, the infection with WNV was diagnosed in humans and detected in other mammals and birds. Then have been collected 76 blood samples from dogs from Bacau, Suceava, Tulcea and Iasi Counties. As a result of performing the serological screening, seropositive dogs were found in all the counties, showing a wide geographical distribution of this virus in dog populations from households and shelters (Crivei et al., 2016).

In 2017, according to ECDC, there is a slight decrease in human's incidence. If in 2016, there were 93 confirmed cases, in the next year, there were reported 58 confirmed cases and six

deaths. As it was in the previous year, in Bucharest were reported most of cases (n=16) and deaths (n=3), followed by Giurgiu (n=9) and Braila (n=5) Counties.



**Fig. 2** Spatial distribution of human cases in 2017

### Conclusions

WNV is a category B (Bigham AW., 2011) pathogen that debuted as an epidemiological phenomenon in 1955 but had a major impact on public health in 1996. After that outbreak, a national surveillance system was implemented in Romania recording WNV infections. The second major outbreak occurred in 2010, when 57 cases were reported in humans.

After 2010, WNV infections were reported annually, with the evolution trend showing a decrease in the number of cases after this year so that a constant number of cases (11 and 13 cases) could be recorded in 2011 and 2012. In 2013 - 2015, there has been a steady increase in cases, and in 2016, most cases have been reported since the 1996 epidemic (n=96). In the last year surveyed in 2017, a decrease in the number of cases was reported, reported 58 cases.

Spatial distribution of human cases shows a persistent circulation of the virus in the south and south-east sides of the country, known as endemic, but from year to year, it can see the spread of the virus to other parts of the country (the center and the west sides - Sibiu, Mures and Bihor counties). The persistence of viral circulation in the south of the country could be favored by the existence of the Danube Delta, which represents an important area of passing, nesting and feeding for the migrant birds (Prioteasa FL., et al., 2007), and also because of the abundance of the abiotic factors (temperature, rainfall, humidity, the existence of stagnant water) that favors the density and the behavior of the vectors, and also establish the inter-relationships among virus, vectors and vertebrate hosts (Krmer LD., et al., 2016). The spatial distribution of the virus is in line with the European Mosquito Bulletin, according to which, the main vectors of the WNV (*Culex pipiens*) are distributed along the south, southeast, north and central sides of the country (Nicolescu G., et al., 2003), also the high number of cases reported in Bucharest, it might be due to the existence of

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another competent vector for West Nile Virus, the *Aedes albopictus* mosquito, who was detected since from 2012 (Ceianu CS., et al., 2016), confirming also the urban outbreaks.

Regarding demographic aspects, almost in every year, the elderly and the male sex were predictors of developing neuroinvasive disease, but also diabetes, alcohol abuse, heard diseases, hypertension, renal disease appeared to be a significant risk factors for developing WNV infection.

In animals, the WNV infections were detected as a result of the serological screening, the virus being detected in horses, domestic birds, wild boars and dogs. The wide variety of species shows that the virus circulates among animals populations, and also its ability to overcome the species barrier. Also the animals might be used as sentinel for the early detection of the virus in human populations.

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# **Observations regarding in vitro hatching of *Raillietina spp.* (Cestoda: Cyclophyllidea) oncosphere**

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## **Abstract**

The study has been realised in May 2016 having as purpose the observation of the oncosphere hatching process. The eggs of *Raillietina spp.* were aquired from feces belonging to Great Spotted Woodpecker (*Dendrocopos major*) that was naturally infested. Initially, the samples were examined from a parasitological point of view using the Willis method. The egg concentrate has been obtained using the protocol described by VOGÉ and all. (1961) with some adjustments. The egg suspension was incubated at 30°C for an hour. The hatching process of the oncosphere has been observed on the slide with the optical microscope (Motic B series) fitted with a Moticam 1000 camera, using the x400 magnification. Measuring has been realised with Motic Images Plus 2.0 software. The temperature of the slide in the moment of examination was 32°C. The whole hatching proces lasted 5 hours and went through the following stages: after half an hour the hexachant embryo has broken the basal membrane and entered the vitelline layer, the oncosphere was vigorously pushing and scraping the granular structure belonging to the vitelline layer, then the vitelline membrane and the egg shell was perforated after four and a half hours, thus completing the actual hatching process. After hatching, the oncosphere engaged in a series of swim-like motions in the liquid mass.

**Keywords:** *Raillietina spp.*, hatching oncosphere, great spotted woodpecker.

## **Introduction**

The hatching in cyclophyllidean cestodes is defined as the release of the oncosphere by the layers of the egg and its stimulation to activity (life) [8]. The hexachant embryo *in vitro* hatching was observed through the use of proteolitic enzymes (artificial gastric fluid and artificial intestinal fluid) [1, 4, 7], biological extracts from invertebrates in different environments [1, 5, 9] and by increasing pressure to the eggs [6].

## **Material and method**

The present study has been conducted in May 2016. The cestodes eggs were obtained from great spotted woodpecker (*Dendrocopos major*), naturally infected with *Raillietina spp.*. The great spotted woodpecker is a common bird in the deciduous and coniferous forests but also in gardens and parks. It feeds on insect larvae found on trees but also with spruce seeds, or other cereals, and in spring it eats vegetable sap [3]. The fresh feces samples were collected in sterile polyethylene containers and were maintained at refrigeration temperature for 24 hours. Then they were examined using the Willis method.

*The preparation of the egg concentrate* was performed after the method described by VOGÉ et al. (1961), with the following modifications: commercial solution of NaCl 0,9% (pH = 5,5) was used for suspension washing.

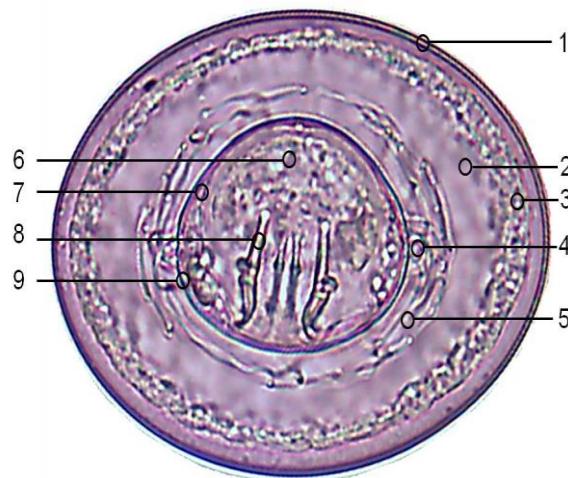
*Experimental protocol:* The egg concentrate was agitated and transferred to 10 Eppendorf tubes of 1 ml and maintained at a refrigeration temperature until examination (+4°C). Then each tube was incubated for an hour at 30°C. One drop of suspension was then examined under the

microscope in order to observe the movement of the hooks and the hatching of the hexachant embryo.

The examination was made using the optical microscop (MoticB series), equiped with a Motic 1000 camera and using x400 magnification. The measurements were made with the Motic Image Plus 2.0 software. The temperature during the examination was 32°C.

## Results

*The hatching process* (fig.1-5). The sequences that precced the releasing of the hexachant embryo of *Raillietina spp.* from all egg structures, starting from the intact egg that presents motility of embryonary hooks (fig. 1) are: (I) the hexachant embryo is breaking the basal membrane (fig. 2), then it leaves the central area of the egg and penetrates the exterior layer (vitelline layer), (II) the increased activity of the onchosphere, that with the help of hooks pushes and grazes vigorously the granular structure of the vitelline layer and vitelline membrane, (III) then it perforates the egg shell (fig. 3), (IV) the true hatching of the hexachant embryo (fig. 4).



**Fig. 1.** Egg of *Raillietina spp.*

- 1 – Transparent shell;
- 2 – Vitelline layer/ Exterior layer;
- 3 – The granular structure of the vitelline layer;
- 4 – Polar thickenings (n=2);
- 5 – Filaments (appears from polar thickenings);
- 6 – Hexachant embryo (oncosphera);
- 7 – Oncosphere membrane;
- 8 – Hooks (n=3 pairs);
- 9 – Basal membrane.

After hatching, the oncosphera swims freely in the liquid mass (fig. 5). The entire hatching process was deployed in an 5 hour interval. Moreover, after approximatively 30 minutes the oncosphera releases from the central area of the egg by breaking the basal membrane and reaches the vitelline layer. Here, the hexachant embryo damages the other internal structures of the egg and after 4.5 hours the true hatching happens. Before the oncosphera enters the vitelline layer, the egg shell is smooth on both sides (internal and external).

**Table 1.** Morphometrics of *Raillietina spp.* eggs.

	L (μm)	W (μm)	T (μm)
<b>Egg</b>	103.73 ± 4.67	77.50 ± 5.36	-
<b>Onchosphere</b>	50.58 ± 1.26	40.04 ± 1.54	-
<b>Hooks</b>	20.86 ± 1.13	-	(D) 2.48 ± 0.27 (M) 4.30 ± 0.34 (P) 1.63 ± 0.21

L – length, W – width, T – thickness, D – distal part, M – medial part, P – proximal part.



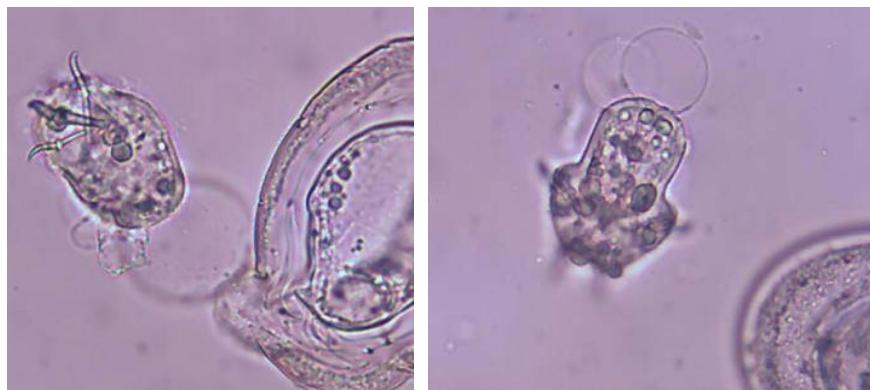
**Fig. 2.** First step of hatching:  
breaking the basal membrane



**Fig. 3.** The onchosphere pushes and  
grazes the internal structures of the  
egg



**Fig. 4.** The true hatching



(a)

(b)

**Fig. 5.** Free and viable oncosphere

## Discussions

The differences between the anatomic and histochemical structure of the cyclophyllidean eggs, may lead to different requirements of incubation fluid [8]. In the case of *Raillietina spp.* eggs, the hatching has been obtained without simulating the conditions found inside the intermediate host. The only physico-chemical stimulation applied to the egg shell was the osmotic shock produced by the saturated solution used in the process of obtaining the egg concentrate.

The effect of the Tyrode's extract of adults or larvae of *Dermestes vulpinus* (pH 7,2 to 7,8) produced the hatching of the oncospheres of *Hymenolepis diminuta* after 15 minutes to half an hour [9].

## Conclusions

The in vitro hatching of the hexachant embryo from the eggs of *Raillietina spp.* maintained in an environment with pH 5.5 and temperature of 32°C, was obtained in approx. 5 hours.

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# **Forensic aspects in animal abuse/cruelty: negative behaviors of humans towards the livestock**

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## **Abstract**

*Abuses (cruelty) are instances of inappropriate treatment of animals by careers or persons interacting with the animal as part of the production process, such as before sacrifice or during shearing. These circumstances are then generalized on a large scale, leading to the spread of this behavior and posing a serious risk to animal welfare. It is important to establish the prevalence of behaviors, the reasons for the occurrence and the appropriate responses from livestock communities. These issues need to be taken into account in the context in which we have a moral duty towards the animals we care for and provide us with consumer goods. In general, the idea is to use animals for the company, food or clothing, an idea embraced even by many who do not consume meat but buy meat products for feeding their pets. Animals that provide these resources are dependent on people for food and shelter throughout their lives. Therefore, we have a duty to care for those animals that depend on us. In our research, the prevalence of deliberate maltreatment of animals in the county of Iasi was monitored. This does not mean that there were no occasions when an individual harmed the animal due to a certain set of circumstances, such as the animal's response to orders or because the person is upset about other reasons or is not feeling well. Cases of animal abuse are numerous, from precarious housing, to starvation, beating and even slaughter in barbaric conditions. Under existing laws (Law 205/2004 modified and republished in 2017) on animal welfare, a person commits the offense of cruelty to animals when the person causes death or unjustifiable physical pain or suffering to any animal by an act, an omission, or willful neglect.*

**Key words:** animal abuse, livestock's, law

## **Introduction**

This paper is about the veterinary forensic findings in animal cruelty cases, this subject has been written very little in Romanian veterinary forensics. It is important to establish the prevalence of behaviors, the reasons for the occurrence and the appropriate responses from livestock communities.

Maybe, a problem is the lack of training in psychology and criminology of veterinarians, as Morgan C. (2005) suggested, they are not prepared to evaluate their clients' abilities to improve the care of animal. So, it is proved that there is a connection between animal cruelty and other acts of violence such domestic violence. (Rollin B., 1998, Reisman R., 2004).

Melinda D. Merck (2007) said that animal cruelty is the act of abusing a helpless victim who has no recourse and no voice. That's why veterinarians must be their voice and seek justice for their suffering.

In our research, the prevalence of deliberate maltreatment of animals in the county of Iasi was monitored. This does not mean that there were no occasions when an individual harmed the animal due to a certain set of circumstances, such as the animal's response to orders or because the person is upset about other reasons or is not feeling well. Cases of animal abuse are numerous, from precarious housing, to starvation, beating and even slaughter in barbaric conditions.

Under existing laws (Law 205/2004 modified and republished in 2017) on animal welfare, a person commits the offense of cruelty to animals when the person causes death or unjustifiable physical pain or suffering to any animal by an act, an omission, or willful neglect.

## **Material and methods**

In our research, the prevalence of deliberate maltreatment of animals in the county of Iasi was monitored.

A number of 23 cases of animal abuse have been reported, including from precarious housing conditions to starvation, ill-treatment and even clandestine slaughter in unauthorized sanitary-veterinary places, during 2015-2016.

Participating institutions were represented by Direcția Sanitară Veterinară și pentru Siguranța Alimentelor (DSVSA) Iasi, IPJ Iasi and Faculty of Veterinary Medicine.

## **Results and discussions**

We will present in this paper three cases of animal abuse that were seen in 2016 in Iasi county and we will try to show the important role of veterinarian's trained in forensics for prosecution preparing phase of the trial.

In locality Holboca, there were discovered inappropriate conditions of animal maintenance, without respecting minimum welfare conditions. The animals were kept in the open field, without a roof during the winter (Fig. 1). Some of the animals were born in that paddock and the fetuses died by frost (Fig. 2, Fig. 3, Fig. 4). The animals were in an advanced state of weakness, kept in misery, dying in the cold and being eaten by stray dogs (Fig. 5).

The case was referred to the Holboca Police Station for the preparation of a criminal case file under Law 205/2004 for the application of ill-treatment to animals.



**Fig. 1 - Inappropriate conditions of animal maintenance**



**Fig. 2 - Calf born in the winter cold**



**Fig. 3** - Unsuitable zoo-hygienic conditions



**Fig. 4** - Dead calf by freezing



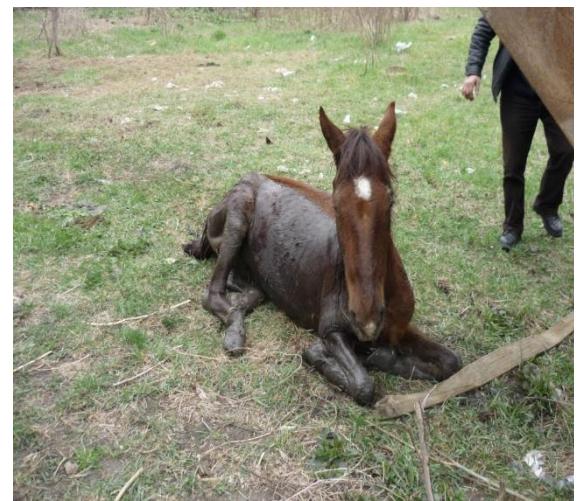
**Fig. 5** - Dead calves eaten by stray dogs

Another case, in the Doi Băieți area of Iași, was found an abandoned horse. It was initially sold to middlemen, then abandoned. The horse was abandoned in a hidden area, surrounded by a hedge, when the people in the area complained of a repellent odor, identified the animal in a state of intense weight loss, malnutrition, numerous areas of necrosis and decubitus plaques on the surface of the body (Fig. 6, Fig. 7).

The animal was unable to stand up or move (Fig. 8). He was euthanized and given to S.C. Protan S.A. (Fig. 9). The owner was sanctioned by contravention, sanction applied by the Iasi Court, under Law 205/2004 an animal protection.



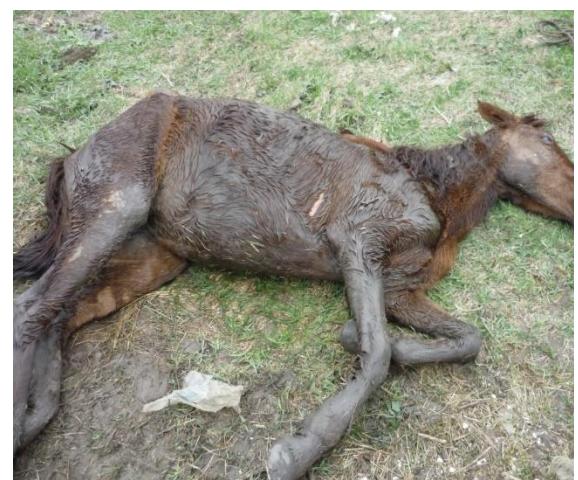
**Fig. 6** – Horse abandoned with necrosis and decubitus plaques



**Fig. 7** – Right part of the body with mud



**Fig. 8** – Impossibility of movement



**Fig. 9** – After euthanasia

It has been discovered an act of slaughtering of horses, in places forbidden by law in another case. The meat from the slaughtering was marketed in public places of Iasi as calf meat. The slaughter point was arranged under an improvised roof with improper pulleys and rudimentary and totally unhygienic slaughtering utensils (Fig. 10, Fig. 11, Fig. 12, Fig.13).

The meat that was packed in bags and prepared for delivery, was totally confiscated under the supervision of DSVSA with Police of Iasi (Fig. 14). The meat was incinerated in the DSVSA Iasi incinerator. The police forwarded the case to the Prosecutor as a criminal complaint.



**Fig. 10 - Slaughter point**



**Fig. 11 - Unhygienic slaughtering utensils**



**Fig. 12 - Improper pulleys**



**Fig. 13 - Proof of meat origin: horse**



**Fig. 14 – Bags with the meat prepared**

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## **Conclusions**

Most of the actions performed were mixed team actions with representatives of DSVSA and IPJ Iasi, according to the collaboration protocol with this institution.

Owners were drawn up criminal files that were submitted to the Iasi Prosecutor's Office for settlement.

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# **Review of goat breeding and herds health status in Romania**

## **Évaluation de l'état de santé de troupeaux des chèvres en Roumanie**

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### **Abstract**

The interest in goat breeding in Romania is growing, our country ranked fourth in Europe, following countries with tradition in the field, such as United Kingdom, Spain and Greece. The goat population grew from 971830 animals in 2006 to 2057309 in 2017. As breeds, the most common are Carpathian and White Banat native goats, followed by French Alpine and Saanen, imported lately to improve milk production. To date, in Romanian literature had been reported researches on goat livestock development, optimization of breeding technologies, genetic improvement of milk production in Carpathian breed, evaluation of physical and chemical parameters of milk and meat, evaluation of goats reproductive function, etc. On the subject of diseases and health problems in goats, there are studies on white muscular disease, paratuberculosis, contagious ecthyma, infectious respiratory disorders, gangrenous mastitis, contagious agalactia, bluetongue, caprine arthritis and encephalitis, and parasitic infections as coccidiosis, tapeworm (*Monezia, Cestoda*) and round worms (*Nematode - Trichostrongylus sp.*) infestation, etc. The aspects of health problems in goat livestock, diagnosed and published during the last years, are few and constantly changing, unsystematic and insufficient, even though in Romania there is a national surveillance and control program for goat health status.

**Keywords:** goat, livestock, health status

### **Introduction**

The aim of the present paper is to analyze the goats heath status in Romania and to present the reported data on the subject of diagosed diseases. From this point of view, it should be noted that in many areas of the country goat rearing enjoys attention from small farmers, given that the number of goats is increasing. The goat population grew from 971830 animals in 2006 to 2057309 in 2017. As breeds, the most common are Carpathian and White Banat native goats, followed by French Alpine and Saanen, imported lately to improve milk production.

In Romania the main goal of goat's breeding is meat production, especially kids and milk production. Goat's milk is commonly processed into cheese, and there is increasing demand of dairy industry for it. Goat farming is mainly organized into mini farms (30-60 goats), whose main product is milk, that is usually processed into cheese in family farms. Better use of underutilized pastures, the country holds 4.9 million hectares of pastures mostly situated in regions listed as less favoured areas, indicates significant development potential of goats in Romania. It requires a lot of work, with a commitment to increase the goats population. Significant investments in modernization and wider use of new technological procedure will raise productivity, improve welfare and quality of better monitoring the health of individual animals and goat herds and increase economic efficiency. An effective animal health program is an essential part of a successful dairy goat management program.

### **Metabolic disorders**

Iodine deficiency determines the appearance of goiter, which is a characteristic of thyroid gland enlargement in all domestic mammals, birds and reptiles (Osame S. et al., 1994; Corradini P. et al., 2000; Garner M.M. et al., 2002; Fyfe J.C. et al., 2003). Sometimes, iodine deficiency

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during pregnancy could be the cause and the kids surviving after birth develop goiter in later stages of life (Vijlder D., 2003). Goiter in utero is caused by either primary or secondary iodine deficiency (Maxi M.G. et al., 2007).

Among the incriminated cases in the appearance of kids goiter is the feeding of pregnant females with low iodine feed or goitrogenic compounds which interfere with thyroxinogenesis (brassica plants, soybean byproducts and water with high content of calcium and nitrates) (Blood D.C. et al., 2000; Radostits O.M. et al., 2007; Sing R. and Beigh S.A., 2013).

The last outbreak reported in Romania regarding iodine deficiency in kids, was recorded by Mihai I. et al. (2018) within an Anglo Nubian goat farm. According to Mihai I. et al. (2018), the pregnant goats were fed with goitrogenic plants (cabbage), but there was no palpable enlargement of the dams thyroid glands. Clinically, iodine deficiency is characterized by the presence in the upper third of the neck of a submandibular bilobata formation that overlays the trachea just below the larynx, symmetrical, united in the distal part (Liklater & Smith, 1993).

According to Mihai I. et al. (2018), the most common cause of goiter in animals is an iodine deficiency. In order to reduce the economical wastage caused both the therapeutic expenses and an increased mortality rate, Mihai I. et al. (2018), recommended supplementing the iodized salt fodder ration as well as avoiding the goitrogenic feeding of goats in the last months of pregnancy.

### **Toxicologic disorders**

Urea is part of simple fertilizers which contain a single fertilizer element, respectively nitrogen (Mierleșteanu I. et al., 2013). Urea and other non-protein nitrogenous substances have been introduced into ruminant feed as a source of protein nitrogen, whereby about 50% of the required protein for them can be substituted (Mierleșteanu I. et al., 2013).

In Romania, a case of urea poisoning in goats was recorded by Mierleșteanu I. et al. (2013). Thus, 16 goats from a herd of 126 common breed goats were poisoned after consuming urea, used as a fertilizer. According to Mierleșteanu I. et al. (2013), urea was used for crops fertilization and even if the therapeutic intervention was quick, from those 16 goats, 11 of them had a fatal outcome. Thus, according to Mierleșteanu I. et al. (2013), the accidentally consumption of urea by goats produces a hyperacute poisoning with clinical signs and fatal outcome. In the same study, the author claims that in case of accidentally ingestion of high doses of urea, the antidotal therapy becomes ineffective.

In conclusion, Mierleșteanu I. et al. (2013) recommends ruminants breeders that a particular attention is required in case of nitrate substances manipulation used in fertilization, taking into consideration that an accidentally urea consumption can lead to poisoning with serious consequences.

### **Respiratory and digestive disorders**

Of all goat maladies, those affecting the respiratory system can cause substantial loss through high morbidity and mortality (Bordeanu A.D. et al., 2012). Most of the infectious agents that cause respiratory disease are usually common inhabitants of the respiratory system (Emikpe, 2009).

Also, most of carried bacterial flora is found in respiratory and digestive tracts of healthy animals or humans and exerts no pathogenic effect under physiological circumstances. When favoring factors induce immune suppression, apparently harmless strains can become highly pathogenic for animals and consumers or people who work in the animal sector (Bordeanu A.D. et al., 2013).

Regarding respiratory disorders in goats, Bordeanu A.D. et al. (2012) conducted a study on 20 goats raised in extensive conditions (Harghita County, Romania), in order to isolate and characterize bacteria from the nasal passageways of clinically healthy goats.

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Thus, Bordeanu A.D. et al. (2012) identified that bacterial strains which normally are present within respiratory tract of healthy goats raised in shelters, but also bacterial strains with high pathogenicity can cause harm in some optimal conditions. Also, they observed notable differences in bacterial populations in different seasons (winter and spring), and a major cause can be the housing conditions.

In conclusion, Bordeanu A.D. et al. (2012) claims that all bacterial species isolated from goats in winter and spring season have a high risk of pathogenicity in proper conditions. Therefore, is necessary to take safety measures when handling goats and their kids but also to respect proper hygiene protocols for public health in general.

In 2013, Bordeanu A.D. et al. conducted another study regarding identification and comparison of ported digestive and respiratory bacterial strains from goats and sheep cohabiting in a mixed heard from Romania. Thus, the research was carried out on 30 healthy animals (15 goats and 15 sheep) belonging to the same herd (Bordeanu A.D. et al., 2013).

Interestingly, enterobacterial species such as *E. coli* have been identified in the respiratory system of goats, but not of sheeps, while *E. cloacae* strains were present in the respiratory tract of sheep but not of the goats. Although the flock consisted of mixed species, there were differences between the bacterial isolates, suggesting differentiated susceptibility and feeding behavior in these species (Bordeanu A.D. et al., 2013).

However, the bacterial isolates from clinically healthy goats and sheeps could exert pathogenic effects under stressful circumstances, underlining the importance of early identification of pathogens and the accurate sanitary management of the heard (Bordeanu A.D. et al., 2013).

### **Parasitic diseases**

Is well known that goats are very sensitive to internal parasitism, which can cause a decrease in fertility, abortion, an increased susceptibility to diseases and death.

In 2011, Iacob O.C. conducted a study on 1450 Carpathian breed goats, in order to reveal different aspects of their digestive and pulmonary parasitogram. Following examinations, the author claims that *Eimeria* genus was dominant to all age categories, with an extensivity of 90-100% and a value of OPG (oocysts per gram of faeces) between 0-2500.

According to Iacob O.C. (2011), nematodes of the family *Trichostrongylidae* were dominant in adult goats, with an extensivity of 100% and an OPG value between 2000-5100, which defines a strong infestation; in young goats and bucks (treated against parasites two weeks previous to going out to pasture), trichostrongillids had an extensivity of 0-10% and a 0-50 OPG value.

Pulmonary nematodes of the *Protostrongylus* genus and *Protostrongylus rufescens* species were dominant through unique infestation in adult goats and pregnant youth (Iacob O.C., 2011). Trematodes of the *Dicrocoelium* genus and *Dicrocoelium lanceatum* species were represented by rare invasional elements identified in young individuals and adult does (Iacob O.C., 2011).

Finally, Iacob O.C. (2011) claims that a medical prophylaxis applied selectively in the herd according to age and short-term economic interest, contributes to the infestation and disease of animals at their first grazing, to the parasitical pollution of grazing areas and to the recording of much higher economical losses.

### **Infectious diseases**

Researches on contagious ecthyma (Sore Mouth) in goats were described in many counties of Romania. This infectious disease caused by an epitheliotropic virus (ORF, family *Poxviridae*), has usually an acute evolution. Clinically, ecthyma is characterized by a vesicle-pustular eruption, with predominant perribucal and mouth localization, rarely being seen localization on foot, mammary gland and genitals. The disease was first recorded in Romania by Riegler in 1935, near Bucharest. Latest epidemiological investigations in Romania (during 2008-2012) on Sore Mouth

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were made by Rusu R.O., highlighting the circulation of ORF virus in goat herds from Iași and Botoșani Counties, using molecular methods for viral detection and characterization (Rusu RO et al., 2014). The same author studied the efficacy of vaccine protection (Scabivax) on goat herds: in kids and adults (Rusu RO et al., 2014).

Studies on contagious ecthyma in Braila and Giurgiu Counties or undertaken by Constantin T. et al. (2012) were lesions observed only in young goats (12 out of 84). Lesions were located on lip's skin (100%), muzzle (91.66%), ears (66.66) and feet (50%). In Bistrița Năsăud County investigations were made by Bița et al. (2010) revealing that contagious ecthyma was affecting approximately 30-40% of lactating sheep and goats herd, leading to significant economic losses through decreased milk production and weight loss. Disease evolved throughout the year, but was prevalent during summer season, especially if the pastures are not changed annually. Also this disease had a higher incidence within goats population, which seem to be more sensitive.

Another infectious disease prevalent in goats reared in small farms is gangrenous mastitis caused by *Staphylococcus aureus*. Romania pays special attention to development of livestock sector growth through the introduction of goat breeds with high milk production and genetic improvement of indigenous characters. For this purpose, there were created optimal conditions for maintenance and proper nutrition, and the genetic potential of animals is scientifically directed towards high yields of milk and meat. These, would greatly increase profitability if the morbidity and mortality due to udder disease were reduced. The main consequence of an incorrectly milking is mastitis. These are inflammation affecting the secretory epithelium, the lining of milk ducts and interstitial tissue. Microbial etiology of mastitis in goats includes a wide range of bacterial species, the most important being *Staphylococcus spp.* and *Mycoplasma Spp.* Gangrenous mastitis, caused by *Staphylococcus aureus*, is the most severe reported in goats, resulting in the animal's death or in incomplete or partial sloughing of the udder. In Romania, recent studies were made by Velescu et al. (2009) and Tudose A. et al. (2010) highlighting the importance of the early treatment of this infection. Tudose A. et al. (2010) also evaluated the immune response after vaccination against gangrenous mastitis, by dynamic research of serum protein fractions in goats in vaccinated and unvaccinated groups, revealing the increasing concentration of  $\gamma$ -globulins and decreasing of albumin levels.

A common infection of the respiratory tract of goats throughout the world is parainfluenza type 3 (PI-3). As with other respiratory viruses, PI-3 virus infection impairs the function of the alveolar macrophages and destroys cilia on the bronchial mucosa. This compromises the animal's natural clearance mechanism (defense mechanism) for removing pathogenic organisms from the lower respiratory tract making them susceptible to secondary bacterial infection particularly *P. haemolytica* infection. Uncomplicated PI-3 virus infection doesn't appear to be an important cause of death, but it may result in death because of bacterial pneumonia frequently in kids. In Romania Aniță et al., (2015), tested the immunodetection suitability of viral antigens in routinely fixed tissue specimens as a diagnostic tool for PI-3 infection in goats. Results of this study demonstrate that PI-3 infection should be considered as a possible cause of pneumonia in goats, along with respiratory syncytial virus and bacterial infections (*Mycoplasma* and *Mannheimia spp.*).

Caprine arthritis-encephalitis (CAE) is a goat viral disease caused by a lentivirus belonging to the Family *Retroviridae*. The virus induces a persistent infection by incorporation of the CAEV genome into the DNA of host cell. The monocyte-macrophage cells are the main target of this virus. In clinical cases were described arthritis, mastitis, pneumonia, weight loss and encephalitis. Investigations on this infection in Romania were conducted by Gurău M. et al. (2015) in southeastern Romanian farm and by Mihai I. et al. (2017) in northeastern counties. The investigation results in southeastern farm revealed a high prevalence of CAEV-infection (38.46%),

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proved by serological investigation (active surveillance by ELISA-Ab exams), associated with low clinical cases of CAE, supporting the assertion that most of CAEV infected animals remains asymptomatic. The second study (Mihai I. et al., 2017) purpose was the detection of CAEV antibodies among goat populations from Vaslui County. During 2014-2016, blood samples were collected both from healthy animals and with clinical signs of disease. All serum samples were tested for CAEV antibody by agar gel immunodiffusion (AGID) test. The results of the investigation revealed a 31.86% (94 out of 295) seroprevalence.

Regarding the bacterial infection detected in goats with high economic impact because of the lack of treatment, is paratuberculosis (Johne's disease). *Mycobacterium avium* subsp. *paratuberculosis* (basonym *M. paratuberculosis*) is the etiological agent of a severe gastroenteritis in goats, considered to be one of the most serious diseases affecting ruminants. In Romania studies on paratuberculosis in goats were undertaken by Papastergiu D. et al. (2009) and Macovei I.I. et al. (2012). The serologic investigation of paratuberculosis in small ruminants in Cluj County revealed in 2007 an annual prevalence of 1.28% and in 2008 an annual prevalence of 1.72%. Out of 83 Veterinary District from County Cluj, in 2007 positive cases were detected in 55 (66.26%), and in 2008 in 56 (68.7%). Studies made on dynamic of goats paratuberculosis in Iași County (Macovei I.I. et al, 2012) revealed a seroprevalence of 33,33%.

An effective animal health program is an essential part of a successful dairy goat management program. Good feeding and breeding will not result in maximum production if goats are not kept in good health. Goat's herd health starts with a detailed plan designed to keep the herd healthy and free of disease and debilitating conditions. It is more economical and effective to prevent a disease than to eliminate it by treatment. Overall herd health is the cornerstone to an effective herd health plan. The goal of a preventive based program is to prevent the introduction of pathogens or hazards, and to prevent their spread. The best way to accomplish this is to ensure that the majority of the herd is protected so that disease organisms cannot get a foothold into the herd.

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# The neurotoxic effect of deoxynivalenol in chronic intoxication of chickens

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## Abstract

*Deoxynivalenol (DON) is a mycotoxin produced by species of the Fusarium spp. that infects cereals such as corn, wheat, oats, barley, rice etc. stored in improper conditions. DON has a cytotoxic effect, affecting animal and human health, causing symptoms such as nausea, vomiting, diarrhea, abdominal pain, headache, dizziness, and fever. Forty ROS 308 broiler chickens were subchronic intoxicated with DON in order to observe histological changes in the cerebral cortex and cerebellum. Following the histological examination, the cerebral cortex and the cerebellum of the experimental lot showed a variable spongy appearance, abnormal aspect neurons, with highly acidophilic, granular, vacuolized cytoplasm and pycnotic nuclei. Also, in neurons from the cerebral cortex, loss of shape and loss of the layers arrangement was observed. These changes explain the altered clinical status of chickens from experimental group throughout the experiment.*

**Keywords:** deoxynivalenol, chickens, chronic intoxication, neuronal degeneration

## Introduction

Deoxynivalenol (DON or vomitoxin) is a trichothecenic mycotoxin causing loss of appetite, anorexia and weight loss (Pieters et al., 2002), immunosuppression (Solcan et al., 2012). Laboratory studies have demonstrated that anorexia appears from the first day of DON oral administration (Amuzie et al., 2011, Hattori et al., 2011), or by intraperitoneal injection (Flannery et al., 2012). Acute toxicity studies have demonstrated the presence of toxin in the plasma, liver, spleen, brain only 5 minutes after inoculation (Pestka et al., 2008). In short-term and sub-chronic exposure, the decrease in feed consumption and body weight was observed (Pestka, 2007). A chronic toxicity study demonstrated reduced daily consumption, decreased of total weight, and increases in IgA serum levels (Iverson et al., 1995). In farm animals, the presence of DON has been reported in organs and tissues such as liver, kidneys, muscles, dorsal fat (Doll et al., 2008), eggs (Sypecka et al., 2004), milk (Keese et al., 2008). Considering these and the resistance of vomitoxin at high temperatures of 170°C to 350°C (Hughes et al., 1999), there is a risk of intoxication in humans by consumption of contaminated products.

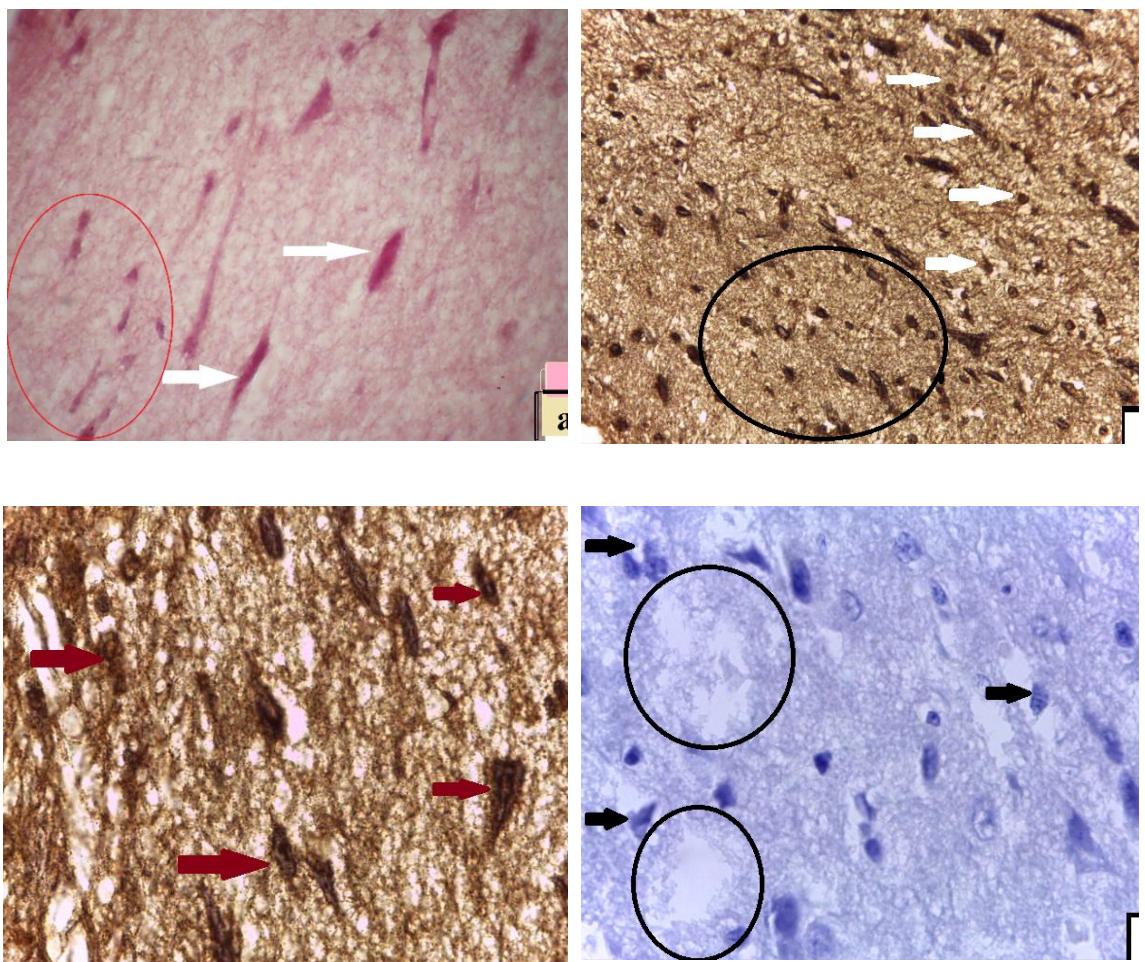
## Materials and methods

The effects of DON on 40 Ross 308 broiler chickens were studied. They were acclimatized for 6 days, then weighed and divided into 2 lots: a control group of 10 chickens and an experimental one of 30 chickens. The animals in the experimental lot were exposed to a dose of 50 µg / kg body weight of deoxynivalenol in sterilized sunflower oil suspension. Mycotoxin administration was performed by gavage for 36 days. Chickens from the control group received only sterilized sunflower oil. At 42 days of age they were weighed, slaughtered and sampled for blood, organs, respectively segments from the encephalus and cerebellum. The pieces were paraffin embedded, sectioned in slices of 5 µm using the Slee Meinz CUT 5062 microtome, then stained by HE, PAS and Gomori methods and examined with Olympus CX41 microscope.

## Results and discussions

Ischemia accompanied by the presence of abnormal neurons and a reduction in the number of these neurons was observed in the cerebellum and cerebral cortex.

Neurons in the cerebral cortex showed changes in size, but also in the cytoplasmic staining affinity, appearing intense acidophilic, granular or vacuolar in PAS staining (fig 1a). Betz neurons showed an atypical smaller aspect, loss of the characteristic layout of the layers (fig. 1b). The nuclei of the modified neurons were larger in size and had an eccentric position (Fig. 1c). Neuronal degeneration was indicated by free spaces without neurons (fig. 1d).

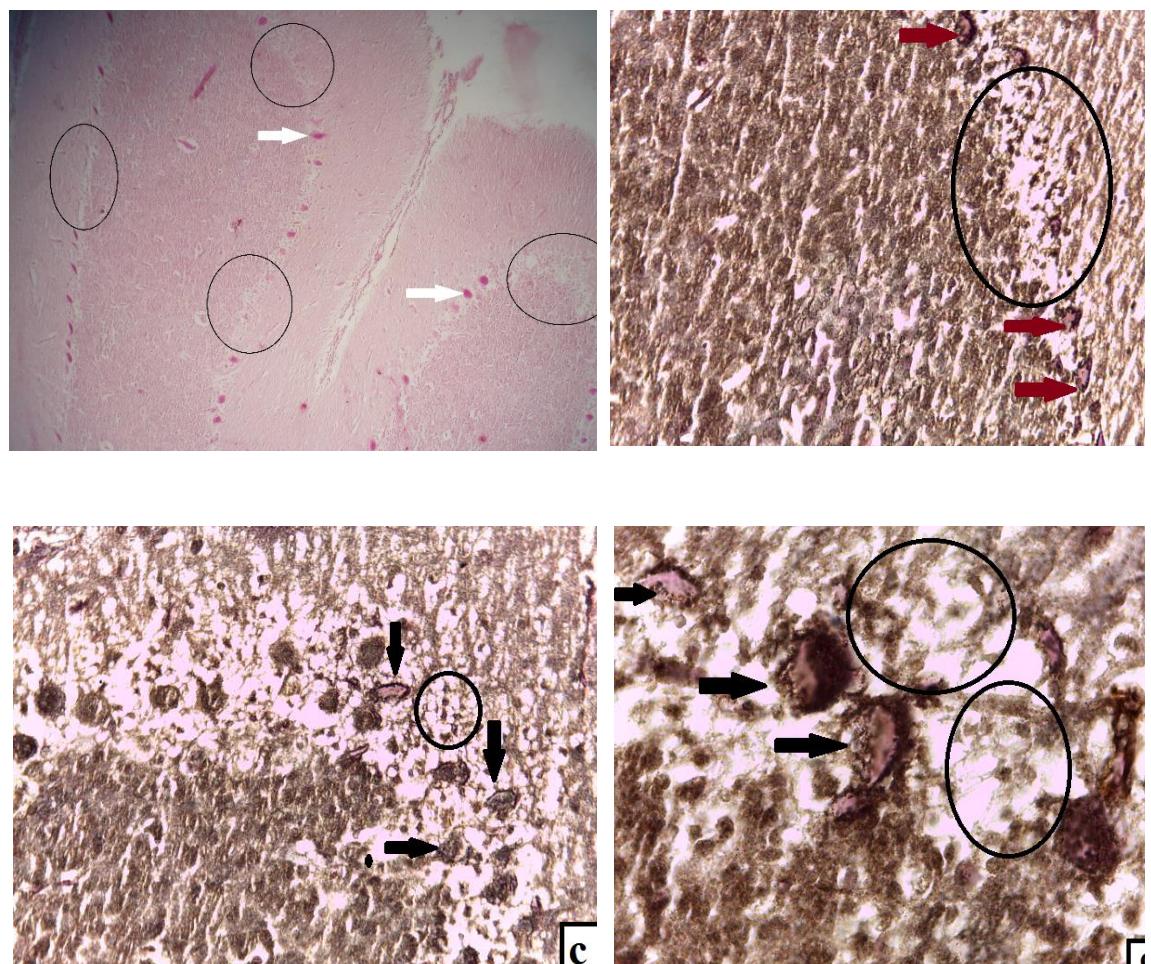


**Fig 1. Cerebral cortex.** A . Rare intense acidophilic neurons, smaller than normal (red cercle). PAS stain x400. B. A bigger number of astrocytes and small Betz neurons with abnormal smaller nuclei (black arrow) and neuronless regions, caused by neuronal degeneration (black circle) Gomori stain x400. C Betz neurons with abnormal bigger, eccentric nuclei (red arrow) Gomori stainx900. D. Abnormal smaller neurons (black arrow) and neuronless regions, caused by neuronal degeneration (black cercle). HE stain x900.

In cerebellar cortex were also observed lesions for suggestive for neurodegenerative effect of DON. So, Purkinje neurons were smaller, intensely acidophilic, containing granular intracytoplasmatic corpuscles

Any layer of cerebellum showed large regions where Purkinje neurons were absent (fig 1a).

An increased number of astrocytes around the Purkinje neurons was observed (Fig. 2b). Their role could be interpreted as a protection reaction from deoxynivalenol action. The degeneration and decrease of Purkinje neurons number was also observed (Fig. 2a, b,c). The nuclei of the modified neurons were larger in size and had an eccentric position (Fig. 2d).

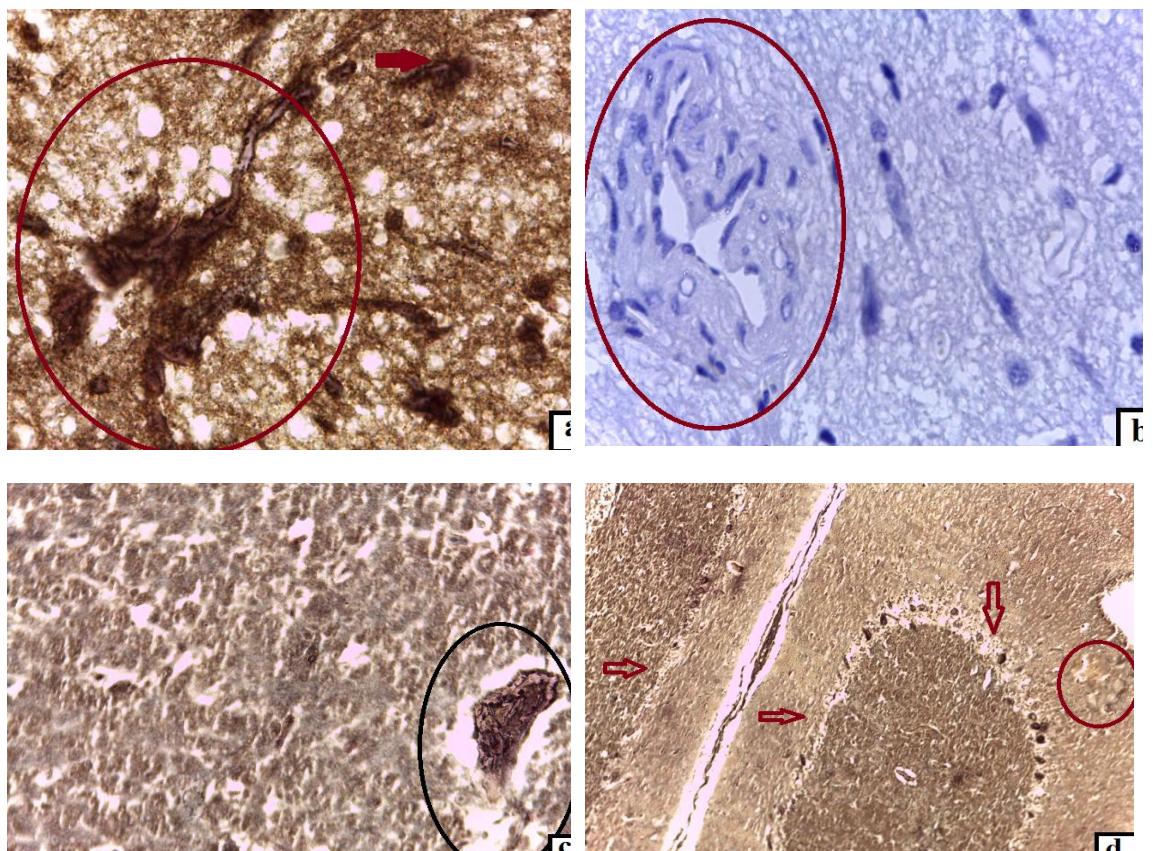


**Fig. 2. Cerebellar cortex.** A. Rare intense acidophilic neurons (white arrows) majority with smaller dimensions and neuronless regions (black cercle). PAS stain x200. B. Many astrocytes (black cercle) and smaller Purkinje neurons with bigger eccentric nucleus (red arrow). Gomori stain x400. C. Purkinje neurons with bigger degenerated eccentric nuclei. Gomori stain x400. D. Abnormal smaller neurons with big eccentric nuclei and many intracitoplasmatic granules (black arrow) and neuronless regions, delimitated by microglia and astrocytes. HE stainx900.

Vascular lesions were observed both in cerebellar cortex (fig 3a, b) and into the cerebellum (fig 3 c, d), consisting in intravascular blood clots (fig 3a), arteriolar degenerations (fig 3b), pericapillary edema (fig 3c) and zones of necrosis into molecular layer of the cerebellum (fig 3d).

No histological changes of the brain or cerebellum were seen in the control group subjects.

In our study we consider that neuronal degeneration characterized by changes in dimensions, vacuolization, the granules presence in the cytoplasm, changes in staining affinity, appearance and position of the nuclei occurred consecutively to a state of chronic hypoxia caused by DON intoxication.



**Fig 3.** A. Cerebral cortex. Intracapillary blood clots (red cercle) and smaller neurons (red arrow). Gomori stain x900. B. Degenerated arteriola (red cercle) and smaller Betz neurons. HE stain x900. C. Cerebellum granular layer. Blood clot in a capillary vessel and edema arround it. Gomori stain x900. D. Cerebellar blade showing absence of Purkinje neurons (degenerated) (red arrow) and coagulation necrosis in molecular layer (red cercle). Gomori stain x100.

DON has the ability to cross the blood-brain barrier and to affect the central nervous system (Girardet et al., 2011a, Maresca M., 2013) increasing the level of proinflammatory cytokines such as interleukin 1 $\beta$ , interleukin 6, necrosis factor  $\alpha$  (Bonnet et al., 2012, Girardet et al., 2011b, Tominaga et al., 2016). DON has been shown to have the ability to induce cellular apoptosis (Wang et al., 2016, Deng et al., 2016). The purpose of this study was to observe the histological aspects of cerebral cortex and cerebellum caused by chronic DON intoxication in chickens.

Long-term administration of this mycotoxin has progressively disrupted the neuronal metabolism, the membrane transport, altered intracellular organelles and triggered the release of lysosomal proteinases - aspects described in the literature (Pestka, 2010, Maresca, 2013).

Modifications have completely destroyed the neuron, causing its disappearance and the spongy aspect of some areas in the microscopic field. Thus, administration of DON determines the same reaction from neurons as in the case of inflammatory factors, especially interleukin  $\beta$ 1 (Girardet et al., 2011a, Bonnet et al., 2012, Langhans, 2000, Clark et al., 2015).

Histopathological aspects showed significant changes in both neuronal hyaloplasma and cellular organelles. The role of mitochondria in cell death in DON intoxication was suggested by highlighting mitochondrial factors such as the pro-apoptotic Bcl-2 proteins and cytochrome C (Wang et al., 2016; Deng et al., 2016; Ren et al., 2015).

The most sensitive to hypoxia appear to be neurons from the surface of the gray matter where associative processes take place (Girardet et al., 2011a, Flannery et al., 2012).

## Conclusions

Oral administration of 50 micrograms/kg b.w DON in chickens for 36 days caused histological changes in the central nervous system correlated with changes in the food behavior. Microscopic aspects were: atypical neurons, variations in size, cytoplasmic granules, nucleus changes, and apoptosis in both the cerebral cortex and the cerebellum.

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# Histological structure of the ovary in adult Zebra fish

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## Abstract

The reproductive system in Zebra fish is intensely analyzed in research, especially in toxicological studies. Therefore, thorough knowledge about normal histology is vital. In our study we have examined 30 adult Zebra fish females with the aim of description the normal morphology and oocytes stages of development. For the gonads examination, histological samples were realized by cutting the fish in cross section or longitudinal section. Samples were fixed in Bouin, embedded in paraffin, sectioned at 5µm, H&E stained and examined at light microscope Olympus CX41. Oocytes in different stages of development were differentiated, measured and main structures were noted. Primary oocytes were mainly disposed in clusters, had small diameter, intense basophil cytoplasm, big nucleus and multiple nucleoli, whilst the cortical alveoli oocytes had larger diameter, germinative vesicle highly irregular, with pleiomorphic and multiple nucleoli and mainly the formation of cortical alveoli with different forms and sizes. In the vitellogenic stage, the oocytes accumulate vitellogenin, a yolk-precursor protein, by endocytosis in membrane limited yolk bodies. The maturation stage of oocytes includes the fusion of the yolk bodies, the nucleus migration toward the oocyte periphery and nuclear envelope breaks down.

**Keywords :** Zebra fish, *Danio rerio*, ovary, histology

## Introduction

Zebra fish is part of the Cyprinidae family and is originated from India and Pakistan. It's intensely used as animal model in research because of the relative facility of its maintenance, reduced development period from embryo to sexually mature adults (3-4 months), low costs of purchase and maintenance, but most important, a remarkable similitude of zebrafish's genome with human genome and high susceptibility at mutagens, carcinogens, teratogens and toxics (1).

Gonads in adult females of zebrafish are lobated and contain a reduced quantity of stroma. The ovaries are structured in two lobes, being located in the abdominal cavity, beneath gas bladder. Considering the fact that in zebrafish the oogenesis is asynchronous, in its ovaries can be found oocytes in all the development stages. Oocytes development in fish can be divided in two stages: a stage of growth and a stage of maturation. In the growth stage, vitellogenesis is highly important, meanwhile in maturation stage can be noted the migration and break down of germinal vesicle, fusion of fat globules with yolk bodies and the release of the first polar body (2).

## Materials and methods

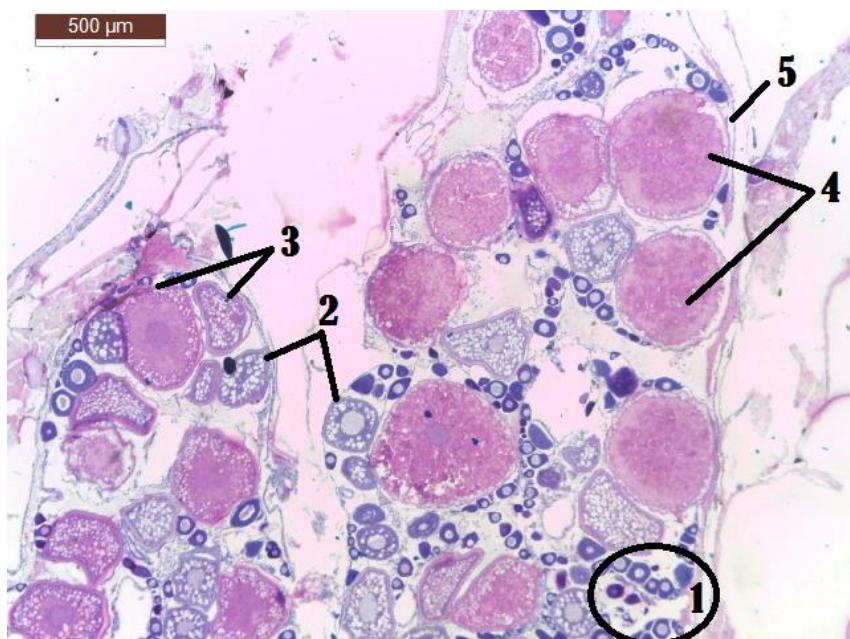
30 females of zebrafish were examined for establishing the development stages of the oocytes. The fish were euthanised using an overdose of propofol combined with lidocaine (3). As a fixation method, we used next protocol: we sectioned the abdominal wall with a surgical scissors from the urogenital pore to the heart without penetrating the heart cavity because the blood will alter the histological results. Whole fish were introduced in a 10% formaldehyde solution for one hour, after which they were cutted by cross or longitudinal (sagittal or coronal plane) sections. The obtained pieces were immersed in Bouin solution for 48 hours. Dehydration was performed by transferring the pieces in 96° alcohol for 24 hours and after in 3 baths of absolute alcohol for one hour each. Samples were cleared with xylene for one hour; paraffin wax embedding was realised by introducing the pieces in 3 successive paraffin-wax baths for one hour each; then microtome

sectioned 5 µm; stained with usual hematoxylin-eosin. The histological sections were examined at light microscope Olympus.

### Results and discussions

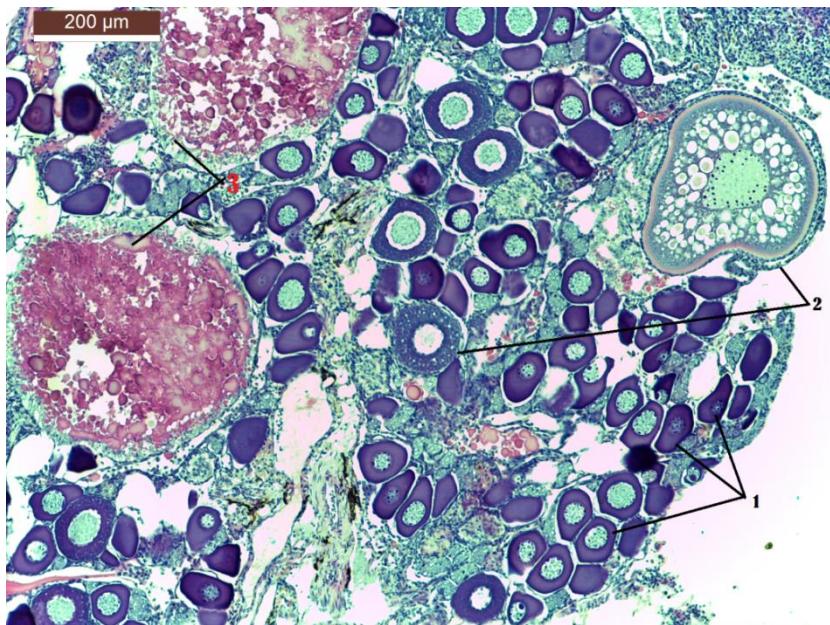
The ovarian sections presented oocytes in all the development stages, differentiated by size and morphological aspect. The oocytes diameter values was calculated for every stage.

Primary growth oocytes were distinguished through their large nuclei with multiple nucleoli and a highly intense basophilic envelope. They were disposed mainly in clusters, dividing all the oocytes from a cluster one single follicular cells layer, but in the late primary growth stage every oocyte presented a thin layer of follicular cells and thecal cells, the nuclear envelope became irregular and the nucleoli were pushed to the side, cortical alveoli begining to form and the primary follicles were scattered in the ovarian mass. The oocytes diameter was comprised between 10 and 80 µm (fig. 1, 2).



**Fig. 1.** Ovary in cross section x40. All stages oocytes can be observed.

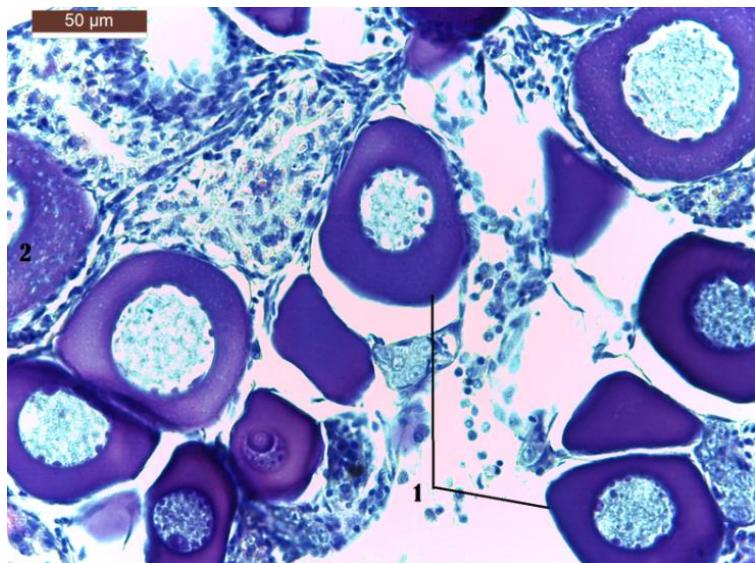
1- primary oocytes; 2- cortical-alveoli oocytes; 3-vitellogenic oocytes;  
4- mature oocytes; 5- ovarian albuginea , H&E.



**Fig. 2.** Ovary in cross section x100. All stages oocytes can be observed.

- 1- primary oocytes;
- 2- cortical-alveoli oocytes;
- 3- oocytes filled with vitellogenic granules, H&E.

The cortical alveolae stage begins when the accumulation of cortical alveolae starts. In this phase the oocytes increase their volume due to cortical alveolae deposits loaded in polysaccharides and proteins, becoming several times larger during this stage. Therefore, their diameter had values between 80-220 µm (fig.3).

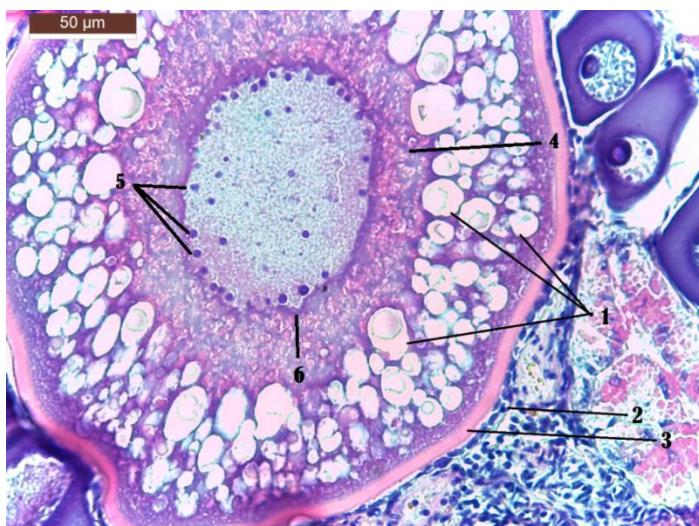


**Fig. 3.** Primary growth oocytes x400. 1- primary oocytes;  
2- oocyte that begin to form cortical-alveolae, H&E.

Nevertheless, the most important growth of the oocytes takes place in the vitellogenic stage when they accumulate vitellogenic granules which contain a protein synthesized by the liver, vitellogenin, and transported in the ovary by endocytosis. The vitellogenic granules are eosinophilic, accumulate in the center of the oocyte and they push to the periphery the cortical alveoli. The nuclear envelope is irregular. The vitellogenic oocytes can measure up to 400 µm (Fig. 4, 5).



**Fig. 4.** Oocytes with cortical alveolae x40. 1- vitellogenic granules; 2- thickening of the vitelline envelope; 3- follicular cells layer, 4-nucleoli, H&E.



**Fig. 5.** Oocyte in vitellogenic stage with cortical alveolae and vitellogenic granules x400. 1-cortical alveolae; 2- follicular cells layer; 3- thickening of the vitelline envelope; 4- small vitellogenic granules; 5-nucleoli; 6- indented nuclear envelope, H&E.



**Fig. 6.** Primary and mature oocytes x 100;  
1- oocytes in primary growth stage; 2- mature oocytes, H&E.

In the maturation stage, the oocytes were filled with vitellogenic granules. The oocyte in the maturation process is surrounded by a granulosa cells layer. The nucleus los its central position due to nuclear envelope break down and will be located at the periphery, but it is barely observed because of the abundant vitellogenic granules and the sectioning process (fig.6). Also, it is difficult to differentiate oocytes in the late vitellogenic stage of the oocytes that had just entered the maturation stage due to the fact that there are no structural histological markers to indicate this differentiation. At this stage, oocytes with a diameter of up to 500  $\mu\text{m}$  are observed.

The final phase is the maturation stage, when the follicle loses the follicular cells layer and is released into the ovary lumen and subsequently into the oviduct and through the urogenital pore, located posterior to the anus, and finally in the external environment.

The literature describes 4 or 5 stages of oocytes growth in Zebrafish. Thus, Selman et al. (1993) divides the development of the ovocytes in five stages: the primary growth stage subdivided into a stage where the oocytes are clustered in nests and a stage in which the oocyte is sheltered by a definitive follicle and enlarges its size; II<sup>nd</sup>stage reveals the appearance of cortical alveolae of different sizes and a thickening of the vitelline membrane; III<sup>rd</sup>stage, of vitellogenesis, is marked by the accumulation of a precursor protein of yolk, vitellogenin, in the form of vitellogenic bodies delimited by their own membrane. At this stage there is an important development of oocytes. IV<sup>th</sup> stage is those of maturation, in which the meiosis is resumed, the germinal vesicle migrates to the periphery of the oocyte, the nuclear membrane breaks, the first meiotic division take place, and the chromosomes move towards the second meiotic metaphase where they stop - at this point the oocyte become an egg. V<sup>th</sup> stage is those of mature egg, ovulation and release into the ovarian lumen. The same results were highlighted in our study.

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Kaviani et al. (2013) describe as the first stage the immature oocyte when it measures between 40 and 60 µm, it is spherical or oval and the ooplasm is best colored by the basic dyes. II<sup>nd</sup> stage is the primary growth stage, when the nucleus is oval or spherical, located centrally, large and with many nucleoli attached to the inner part of the membrane. Oocytes have an average diameter of 78 µm and are surrounded by a thin layer of follicular cells. In the third stage, that of the cortical alveolae, the average oocyte diameter is 215 µm. The nucleoli grow in volume, but they are numerically reduced, the nucleus is large and oval, located centrally. There are large vacuoles near the oolem. The fourth stage of vitellogenesis, with a mean diameter of 295 µm, is characterized by numerous acidophilic vitelogene globules in the ooplasm and migration of the nucleus to the periphery. The last stage, (V<sup>th</sup>), involves an average diameter of 415 µm, the nucleus disappearance, the fusion of the vitelogenic globules and the reduction of zona radiata. The same results have been observed in our study. Özlem Çakıcı et al. (2007) claim the existence of 4 stages: primary growth stage, cortical alveolae stage, vitellogenesis stage and the stage of oocyte maturation. Unlike other authors, they note the migration of the nucleus as occurring at the end of the second stage.

### Conclusions

The ovary of Zebrafish is bilobated, covered by albuginea, and oocytes four stages of development were identified. Dimensions of follicles varies from 10 to 500 µm. The oocyte in the maturation process is surrounded by a granulosa cells layer.

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# Histological structure of the testis in adult Zebrafish (*Danio rerio*)

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## Abstract

The aim of this study was to describe the characteristics of the different germ cells types found in testicles of Zebrafish (*Danio rerio*). Therefore, 30 adult male specimens were sacrificed, sampled and prepared by the usual techniques for light microscopy. The male gonads are paired organs located ventral to the gas bladder and dorsal to the liver. The functional unit of the Zebrafish testis is the spermatocyst, a cluster of clonal germ cells surrounded by cytoplasmic arms of a Sertoli cell. The seminiferous tubules organization in Zebrafish is of unrestricted type, thus spermatocysts form all along the length of the tubule, and spermatozoa are discharged into spermatic ducts. Different types of germ cells were identified into the tubules: spermatogonia, spermatocytes, spermatids and spermatozoa. The biggest cells are the spermatogonia, diploid cells with slightly granular nucleus and pale cytoplasm. They pass through a series of mitotic divisions from which will result the primary spermatocytes, also diploid cells, with intensely basophilic nucleus and reduced cytoplasm. By the first meiotic division results secondary spermatocytes, smaller, haploid cells. The second meiosis produce the spermatids and by their further maturation will result the spermatozoa.

**Keywords :** Zebrafish (*Danio rerio*), testis, histology, germ cells, spermatogenesis

## Introduction

Zebrafish (*Danio rerio*), is a tropical fresh water fish that can grow up to 3-5 cm length as an adult, reaches sexual maturity at 3-4 months old and a female can spawn up to 400 eggs and together with the high similarity of his genome with the human genome and its important sensibility to toxics, carcinogens, theratogens and mutagens makes him an excellent model in medical and environmental research (2, 5).

Spermatogenesis is a highly organized process characterized by sequential transitions of multiple processes: self-renewal of spermatogonial stem cells (SSCs), differentiation of SSCs into differentiating diploid spermatogonia and meiotic events leading to the production of millions of spermatozoa daily (11). This process starts from spermatogonial stem cells, which have the potential for both self-renewal and for differentiating into spermatogonia committed to sperm development. Zebrafish is a juvenile hermaphrodite, with all individuals having ovary-like gonads during early life (1). During the embryonic development, the gonads in Zebrafish undergo an ovarian phase in both males and females. Only starting with the 5<sup>th</sup> week post-fertilization, in males will take place an alteration of the gonad morphology with the decrease in number and size of the perinucleolar oocytes, their irregular shape and intense basophilia and, finally, their degeneration into residual bodies (10). Increased number of gonial cells arranged in cyst-like groups appear. During the subsequent weeks, the male gonads will develop spermatogonia, spermatocytes and spermatids into the seminiferous tubules from the germ cells (3, 4, 8).

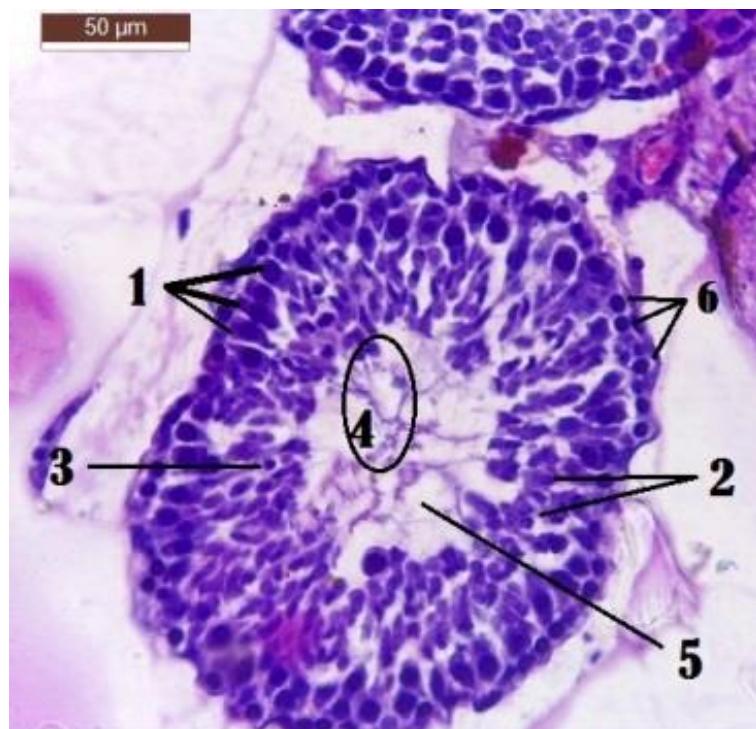
## Materials and methods

30 adult Zebrafish males were taken in study. They were euthanized with overdose of propofol combined with lidocaine (14). The abdominal wall was sectioned and they were introduced in 10% neutral formalin and Bouin solutions. Cross or longitudinal (sagittal or coronal plane) sections through the fish were performed. Samples dehydration was performed by usual method with alcohol series and then cleared with xylene. Paraffin cubes were prepared and cutted

in slices of 5 µm by microtome. For histological examination, sections were stained with hematoxylin-eosin and examined with the light microscope Olympus. The seminiferous tubules with spermatogenesis cells inside (spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and sperms) and Leydig cells were evaluated and most significant parts were illustrated.

### Results and discussions

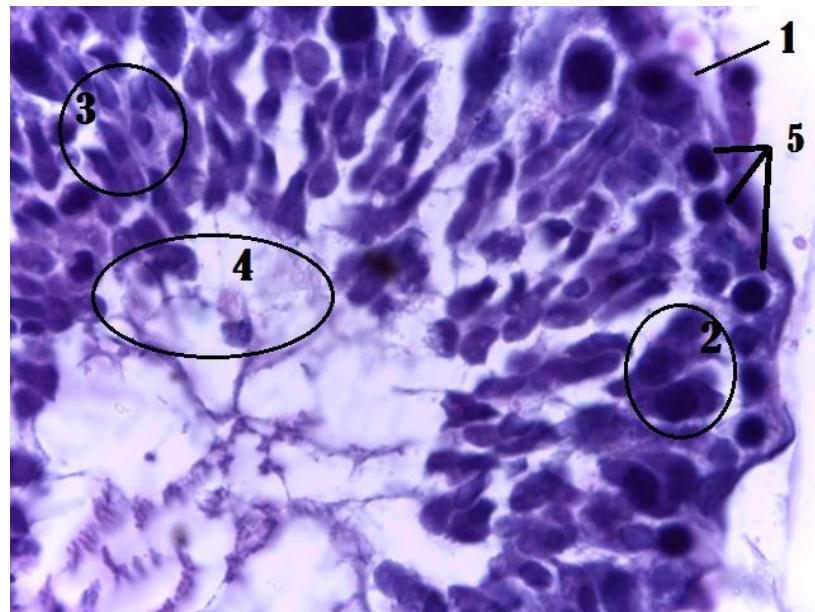
Testicles in Zebrafish are surrounded by peritoneum wall and they are structured by seminiferous tubules, germ cells (spermatogonia primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa), Sertoli cells and also Leydig cells in the connective tissue between seminiferous tubules. The phases of spermatogenesis are divided into spermatocytogenesis, meiosis and spermiogenesis based on the histological characteristics. The specific cells of every phase are identified as different groups of cells in the seminiferous tubules (fig. 1). Four types of spermatogonia, 2 main types of spermatocytes, 3 different types of spermatids and spermatozoa were identified in the seminiferous tubules.



**Fig.1.** Seminiferous tubule in cross section with all stages of germ cells H&E x400;  
1- spermatogonia; 2- spermatocytes; 3- spermatid; 4- spermatozoa; 5- seminiferous  
tubule's lumen; 6- spermatogonial stem cells.

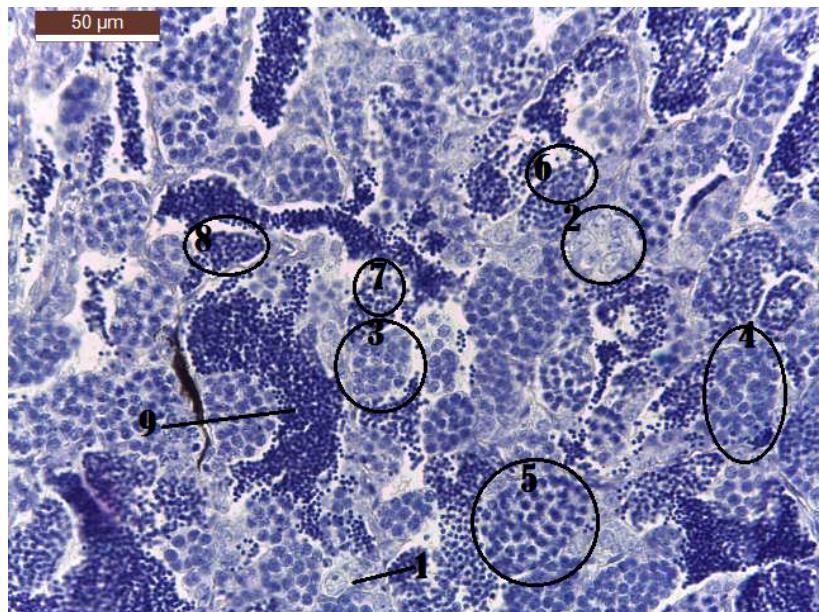
The spermatocytogenesis includes the mitotic transformations of the germinative epithelium. The spermatogonia types differ by their nuclear form, the extent of the cromatin condensation, the number of nucleoli and by cells size. They were the largest germ cells from the seminiferous tubules and they were diploid. Type A undifferentiated spermatogonia were isolated

cells scattered through germinative epithelium with irregular nuclear envelope and elongated, slightly basophilic and low heterochromatic nucleus (fig. 3). They had rich and pale cytoplasm. The type A differentiated spermatogonial cells differed from the undifferentiated ones by the fact that they could be found in groups of two to eight germ cells in a cyst and by their round or oval nucleus with regular envelope. Type B spermatogonia formed groups 16 or more cells arranged in cysts and they had a darker and clearer nucleus than type A spermatogonia due to the high amount of heterochromatin (fig. 4, 5) .

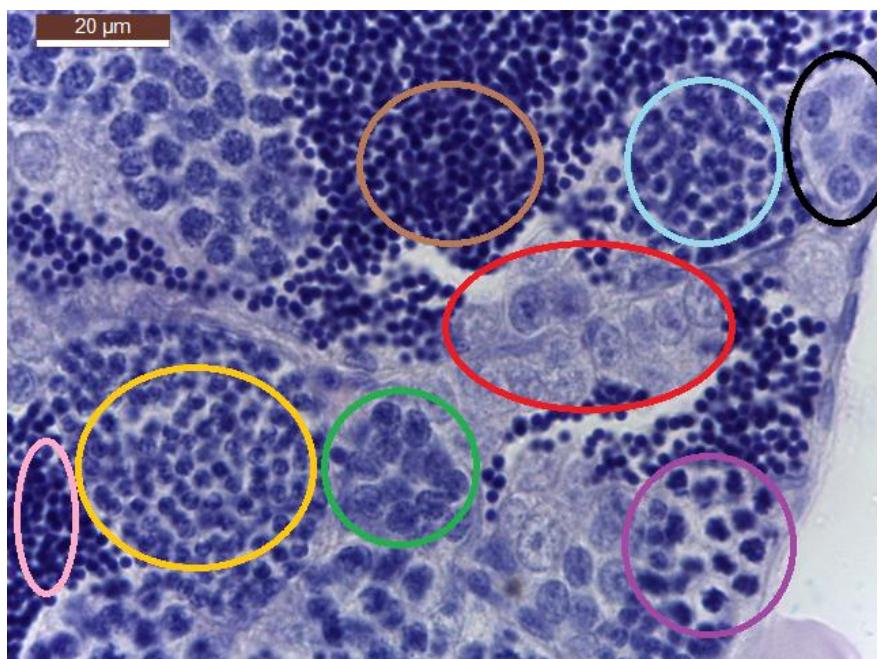


**Fig.2.** Seminiferous tubule in cross section with all stages of germ cells H&E x900;  
1- type A undifferentiated spermatogonia; 2- type B spermatogonia; 3- spermatocytes; 4-  
spermatozoa; 5- spermatogonial stem cells.

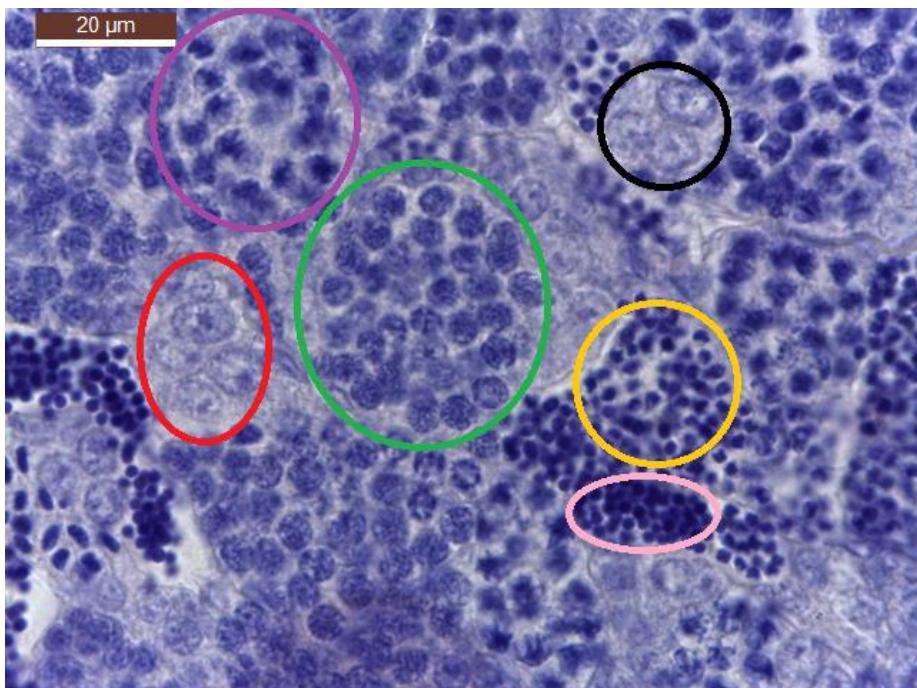
The meiotic phase starts after the type B spermatogonia turn into primary spermatocytes. In this stage that is also called spermatocitary phase spermatocytes in all meiotic phases were founded (fig. 3, 4). At the end of the first meiotic division secondary spermatocytes can be observed. After they pass through another meiotic division, spermatids are formed and spermiogenesis starts.



**Fig.3.** Seminiferous tubule in cross section with all stages germ cells H&E x400;  
1-type A undifferentiated spermatogonia; 2- type A differentiated spermatogonia; 3- type B spermatogonia; 4- primary spermatocytes; 5- secondary spermatocytes; 6- initial spermatids; 7- intermediate spermatids; 8- final spermatids; 9- spermatozoa, H&E.



**Fig.4.** Seminiferous tubule in cross section with all stages of germ cells H&E x900;  
black circle - type A differentiated spermatogonia; red circle –type B spermatogonia;  
green circle –primary spermatocytes; purple circle –secondary spermatocytes; yellow circle –  
initial spermatids; blue circle – intermediate circle; brown circle – final spermatids; pink circle–  
spermatozoa.



**Fig. 5.** Seminiferous tubule in cross section with all stages germ cells H&E x900;  
black circle - type A differentiated spermatogonia; red circle – type B spermatogonia;  
green circle – primary spermatocytes; purple circle – secondary spermatocytes; yellow circle –  
initial spermatids; pink circle- spermatozoa

Spermiogenesis is the final phase and it also may be called differentiation phase. At this point 3 types of spermatids were founded: initial, intermediate and final, according to the nuclear condensation, cytoplasmic reduction and cell size reduction, increase of the space between the cells inside the cyst due to cytoplasmic elimination and flagellum development (fig. 4). The initial spermatids were smaller than the anterior germ cells and more concentrated in the cyst and had a reduced, rounder and condensed nucleus. Intermediate spermatids were even smaller with more reduced and condensed nucleus, scanty cytoplasm and bigger space between them. The smallest cells from the spermiogenesis are the final spermatids that have even more concentrated cytoplasm that will form only a reduced strip around the more condensed nucleus. Larger spaces between final spermatids appear. The completion of the flagellum development, cytoplasmic residues elimination and final spermatids maturation leads to sperm formation. The spermatozoa were the smallest from all the germ cells and were found only in the tubular lumen (fig. 1, 2, 4).

Maack et al. (2003) observed that the alterations of gonadal morphology in some of the 5–11 week-old post fertilization zebrafish are interpreted as a transformation of early ovary-type gonads into testes and that sexual differentiation of developing gonads in fish is considered to be under the control of steroid hormones.

Schulz et al. (2010) has examined the development of spermatogenesis and the testicle structure of zebrafish both with electron and light microscope. He noted that the Sertoli cells could be seen with electron microscope, but not with light microscope and we consider that this observation is related to the fact that we could not see in our sections examined at light microscope the sertoli cells. Thereby, our findings are similar to the data determined in this study.

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Leal at al. (2009) found that undifferentiated type A spermatogonia in zebrafish were distributed along the entire germinal compartment, and so they concluded that zebrafish testis belongs to the unrestricted type. Moreover, they noted that zebrafish testes contain anastomosing tubules, which is another primitive feature. Another observation was that type-A differentiated spermatogonia are originated from previous ones and showed cytoplasmic bridges among them, due to incomplete cytokinesis during mitosis process.

### Conclusions

The testicles in Zebrafish have a different histological structure than mammals. The dividing germ cells are held together by intercellular junctions forming clusters of clonal and synchronously developing cells, structuring the functional unit of the Zebrafish testis, the spermatocyst. This cyst has also in his structure the cytoplasmic processes of Sertoli cells. As the spermogenesis reaches its end and the spermatozoa are formed, the spermatiation process will degenerate the spermatocyst and the sperm will continue their way through the seminiferous lumen, spermatic duct, urogenital sinus and ultimately are released through the urogenital pore (9, 13).

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# Histological aspects of thymus in japonese quail (*Coturnix coturnix japonica*) from one to 180 days of age

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## Abstract

Thymus, described from antiquity, has long time been considered a mysterious organ because its role in the body was unknown. The first microscopic description of the thymus belongs to Hassal, and in the first half of the 20th century the thymectomy was performed to treat "myasthenia gravis." In 1962, the role of thymus in the production of T-lymphocytes which are responsible for the cell mediated immunity was revealed. Bird's thymus represents 2% of the body weight at hatching and continues to develop until the time of sexual maturity. *Coturnix japonica* is a species used in the laboratory as an experimental animal, and it is economically important because of the nutritional value of the eggs which are recommended to be consumed in various diseases. The japonese quail reaches sexual maturity at the age of 7 weeks when signs of thymic involution should be observed. The present study aimed to describe some histological particular features of thymus in the japonese quail. In the study, 25 quail were euthanized at the age of 1, 7, 15, 24 and 60 days for thymus prelevation. The fragments were prepared by paraffin embedding and stained HEA, Giemsa and PAS. This has highlighted some of the particularities of this species. In most species thymus regresses under physiological conditions due to sex hormones action at puberty. The histological aspects obtained in this study demonstrated that in this species the thymus involution was not present even in the individuals sacrificed at the age of 6 months. The dimensions of the cortical and medullary areas of the thymus at 24 and 60 days were approximately constant.

**Key words:** *Coturnix japonica*, thymus, histology

## Introduction

The Japanese quail (*Coturnix coturnix japonica*) is a popular laboratory animal model since the middle of the last century (Huss et al., 2008). Genetic studies regarding the chromosomal and mitochondrial DNA of this species have been conducted (Nishibori et al., 2001, Shibusawa et al., 2004). Because the quail's eggs are used in human alimentation the effects of different diets on fertility, embryonic viability, egg mass and production rates have been studied (Abedi et al., 2017, Mills et al., 1997, Sahin et al., 2003). The influence of diets on lymphoid organs such as thymus, spleen, Fabricius Bursas has also been studied (Sahin et al., 2002, Sahin et al., 2004, Wang et al., 2009). Since thymus is essential for T lymphocytes maturation which are considered to be the cellular immune effectors, the increase in naive B and T cell populations in secondary lymphoid tissues are correlated with increased immunity (Miller, 2004, Wang et al., 2001, Li et al., 2007). In most species the thymus atresia at puberty is determined by sex hormone secretion and the occurrence of necrose areas after estrogen administration was observed in some studies (Razia et al., 2006). The avian thymus is placed around the jugular vein, and histologically, a lobe has a dark cortical area and a light medulla with several thymic corpuscle (Solcan, 2011). Here are double positive cells which after selection and maturation will become CD4+ or CD8+ single positive lymphocytes (Wang et al., 2001). These cells leave the thymus via the blood vessels and reach the peripheral immune system (Bidere et al., 2006, Miller, 2002).

The aim of this paper is to highlight the evolution of histological aspects of thymus in *C. japonica* at various ages.

## Materials and methods

Histological observations were made on several thymuses obtained from 25 quails (*Coturnix japonica*). These were grown in the University Biobasis according to the specific technology. The slaughtering was performed at one day, 7, 15, 24 and 60 days of age, when thymus and various organs were collected. Thymus fragments from 4 birds from each age category were processed by paraffin embedding and stained by HEA, Giemsa and PAS methods. Image capture and measurements were performed with a CX41 Olympus microscope.

## Results and discussions

The histological study of the thymus specimens collected from individuals with different ages allowed the observation and in time evolution. Thus, in certain age groups, particular features of this organ were found in *C. japonica*.

The CX41 Olympus microscope used for image capture also allowed the size measurement of thymus structures presented by age in Table 1.

**Table 1.** Dimensions of thymus structures according to age

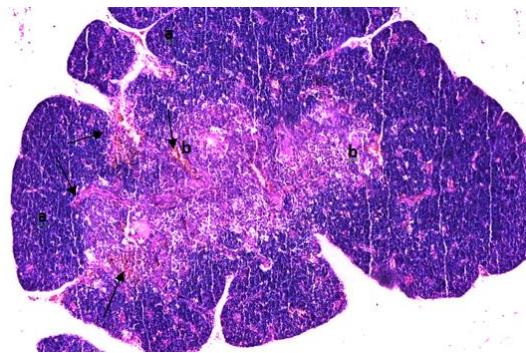
Age /Thymic Structure (μm)	Day 1		Day 7	Day 15	Day 24	Day 60
Thymic lobe	2090-3389		2190-3478	1698-3700	1689-3701	1001-3780
Cortex	750-812		802-1139	425-619	358-620	358-507
Medulla	583-2094		568-2186	581-990	547-989	740-1110
Hassal corpuscle	-		45-334,2	201-210,2	101-209	107-208

The capsule made up of semi-orientated connective tissue showed infiltrations of adipose tissue no matter the age at which the prelevation was performed. Regardless the age, capsulae derived septa with nearly constant diameter splitted the parenchyma of the thymus into lobules.

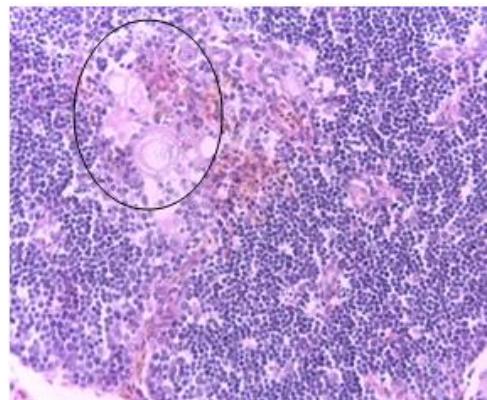
At day one, thymic lobules showed a polygonal appearance, being bound by conjunctival septa with a diametre of 10-12 μm. Cortex (Fig. 1a), consisting of young lymphocytes and epithelial reticulum cells, was intensely colored. At the boundary between cortex and medulla and especially in medulla small congestion areas appeared (Fig. 1, arrows).

The medulla (Fig. 1b) was less colored due to the lower lymphoid population compared with the cortex. The epithelial reticular cells from the medulla showed a polygonal appearance, those located peripherically being smaller than the central ones (Fig. 2). The Hassal corpuscles were atypical, identified as cell clusters without the characteristic concentric arrangement of the cells (Fig. 2, arrow).

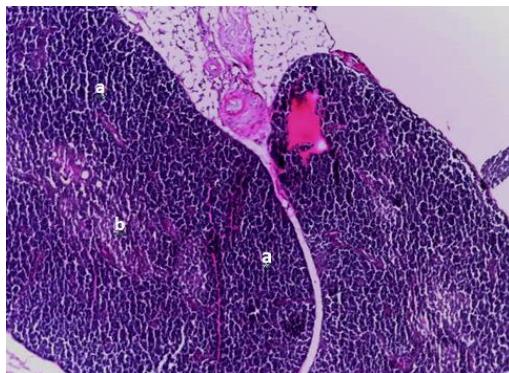
In the 7-day-old chickens, thymic lobules (Fig. 3) showed the same cortex and medulla aspects as in one day-old ones, with the difference that some of the epithelial reticular cells underwent necrosis with the presence of a colloidal central substance, flattened appereance and concentric arrangement characteristic for Hassal corpuscles (Fig. 4).



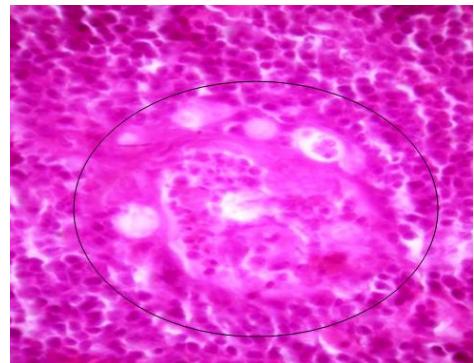
**Fig. 1.** Thymus in one day old quail chicks. The thymic lobe. Intensely dark cortex (a) with immature T-lymphocytes; light stained medulla (b) with mature T lymphocytes and non-lymphocyte support cells. Lobules are delimited by conjunctive septae. Giemsa x 60



**Fig. 2.** Thymus in one day old quail chicks. Enlarged epithelial reticular cells in the medulla. PAS x400.

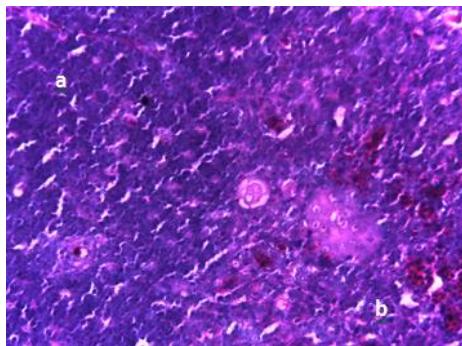


**Fig. 3.** Thymus in 7 days age quail. The cortex of the thymic lobe (a) is evident, dark colored. In the medulla (b) there are clusters of epithelial reticular cells. Giemsa x60.

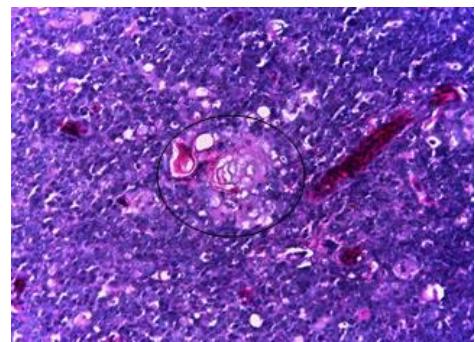


**Fig. 4.** Thymus in 7 days aged quail. The cortex of the thymic lobe is dark colored. In the medulla the Hassall corpuscle consisting of epithelial reticular cells in a concentric arrangement with central lymphocytes. PAS x400.

In quail chickens of 15 days (Fig. 5 and 6) and 24 days of age (Fig. 7) histological aspects are similar to those previously described in the 7 days old subjects, but both the cortex and the medulla were smaller in size.

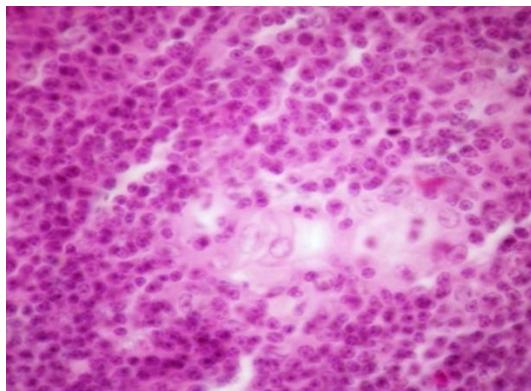


**Fig. 5.** Thymus in 15 days old quail. The cortex of the thymic lobe is dark colored (a). Hassal corpuscle in the medulla(b) consisting of epithelial reticular cells. Capillaries lined with simple cubic epithelium. Giemsa x400.

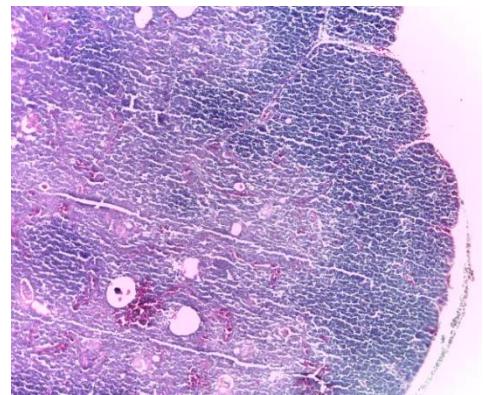


**Fig. 6.** Thymus in 15 days old quail. Dark cortex. In the medulla Hassal corpuscle composed of flattened, degenerated large central cells, with a concentric arrangement.Giemsa x400.

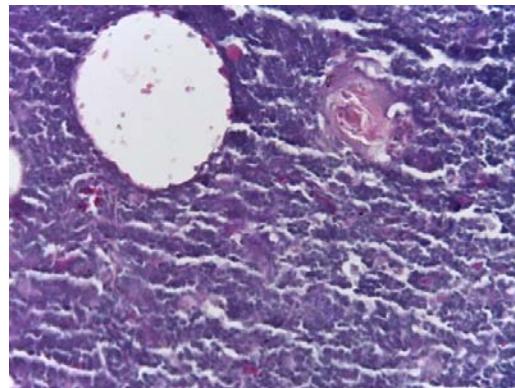
The thymic medulla of the 60-days-old quails contained Hassal corpuscles with areas of necrosis and glycosaminoglycan infiltrations (Fig. 8 and Fig. 9).



**Fig.7.** Thymus in 24-day-old quail. In the medulla Hassal corpuscle composed of flattened, degenerated large central cells, with a concentric arrangement and a few central lymphocytes. PAS x400



**Fig. 8.** Thymus in 60 days old quail. The thymic cortex is obviously dark colored, covered by the delicate capsule infiltrated by fat cells. HEA x400



**Fig. 9.** Thymus in 60 days old quail. Hassal corpuscle in the medulla consisting of flattened concentric arranged epithelial reticular cellswith glycosaminoglycans depositories and lymphocytes. In addition, an area of necrosis is observed. HEA x400

The thymic lobe presented a dense dark cortex with young, large lymphocytes even in the 60 days old quails contrary to other species where involution occurs (Kim et al., 2015). The medial area was lighter colored, consisting in smaller, mature lymphocytes and epithelial reticular cells organized in Hassal corpuscles with various appereances, aspects also described in the literature (Gordon & Manley, 2011, Junqueira & Carneiro, 2005). They consisted of epithelial reticular cells but did not presented the typical concentric arrangement in one day-old quails. The characteristic arrangement was observed only after the age of 7 days. Hassal corpuscles showed specific areas of necrosis and colloidal cystic formations that suggested apoptosis processes at this level which are physiological for intrathymic homeostasis (Huang et al., 2015).

The presence of this phenomenon was highlighted by caspases, proteinases incriminated in inflammation and apoptosis (Deneker et al., 2008, Pozzesi et al., 2013). Causes of thymocytes death were either due to negative selection or due to stress or aging (Godfrey et al., 2000). Researches has shown that T-lymphocytes produced in thymus are apoptosis-oriented if they are not compatible with their future immune function (Alam et al., 1999, Gronsky et al., 2004).

### Conclusions

The thymus in quails younger than 7 days contains Hassal corpuscles with an atypically arrangement of the epithelial reticular cells. The fact that the decrease of the cortex and medulla has not been recorded in thymus collected from the 60 days old individuals and the appropriate values for the 15, 24 days old individuals suggests that the physiological involution of the thymus does not occur in this species.

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## Facial regeneration: current status and perspectives

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### **Abstract**

*Skin aging is a complex biological processes influenced by endogenous and exogenous factors. The primary aim of all skin anti-aging strategies is to reverse the dermal and epidermal signs of photoaging and chronological aging. Healthy and normal functioning skin barrier is an important protector against dehydration, penetration of various microorganisms, allergens, irritants, reactive oxygen species and radiation. The skin barrier may be specifically adjusted to allow penetration of desired substances. Regenerative medicine is the science of replacing, engineering or regenerating human cells, tissues or organs to restore or establish normal form and function. It uses cells, tissues, drugs, synthetic biomaterials and devices to help patients heal or regenerate. The reason of different methods of skin regeneration is to increase skin regeneration, elasticity, smoothness, density, macroscopic and microscopic aspect, changing the skin condition. It is necessary to slow down ageing processes on a cellular level concomitantly. We can provide to the skin primary structural constituents, such as collagen, elastin, to prevent the formation of wrinkles, but some products and techniques do promote the natural synthesis of these substances except elastin enhancing. Simultaneously it is necessary to prevent wrinkle formation by reduction of inflammation (topical or systemic antioxidants) in combination with sunscreens and retinoids to enhance their protective effects. An important attention must be accorded to Transforming Growth Factor-β family, other TGF-β-based approaches (Decorin and Mannose 6 Phosphate), modulation of Smad3/Smad7 Signaling, Epidermal Growth Factor family, Fibroblast Growth Factor family, Platelet-Derived Growth Factor family, Granulocyte Macrophage-Colony Stimulating Factor, Connective Tissue Growth Factor, Interleukin 10, Connexins, other approaches under investigation for scar reduction, collagen, fibronectin, laminin, elastin, glycosaminoglycans and other natural biomaterials. Regenerative Aesthetic Dermatology is focused on innovative treatments to support the skin in restoring and regenerating old and/or damaged tissue and thus improving overall skin quality, promoting faster healing while minimizing downtime and side effects for patients. The onset of effects could be instantly after procedure or can be observed gradually increasing over the time (weeks or months), as we are determining the skin to help itself. The regenerative treatments are encouraging the skin to build more collagen and elastin itself by bio-stimulation: resurfacing the epidermis (topical drug application, ablative LASERs, Laser radiofrequency resurfacing, ablative radiofrequency, microneedling, plasma skin resurfacing, crystal-free microdermabrasion), the formation of new collagen (IPL, Lasers, radiofrequency, infrared, Jett Plasma Medical, medical needling, mesotherapy, platelet-rich plasma, fillers as hyaluronic acid, **HydraFacial MD, Oxygen facial, chemical peels**, hormone replacement therapy, autologous and allogeneic stem cells, gene therapy), slow down the visible aging process by helping in the management of certain dynamic facial lines and wrinkles (Botulinum toxin).*

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*Resurfacing the skin could be considered as a cancer prophylaxis and aesthetic dermatology is contributing to slowly, healthy, gracefully aging.*

**Keywords:** prevention anti-aging, antioxidants, regeneration, rejuvenation, laser, IPL, peeling, fillers, botulinum toxin, plasma, therapy, hyaluronic acid

## **Introduction**

Skin aging is a complex biological processes influenced by endogenous (genetics, cellular metabolism, hormone and metabolic processes) and exogenous factors (chronic light exposure, pollution, ionizing radiation, chemicals, toxins). These factors lead together to cumulative structural and physiological alterations and progressive changes in each skin layer as well as changes in skin appearance, especially, on the sun-exposed skin areas. In contrast to thin and atrophic, finely wrinkled and dry intrinsically aged skin, premature photoaged skin typically shows a thickened epidermis, mottled discoloration, deep wrinkles, laxity, dullness and roughness. Gradual loss of skin elasticity leads to the phenomenon of sagging. Slowing of the epidermal turnover rate and cell cycle lengthening coincides with a slower wound healing and less effective desquamation in older adults. This fact is important when esthetic procedures are scheduled. On the other side, many of these features are targets to product application or procedures to accelerate the cell cycle, in the belief that a faster turnover rate will yield improvement in skin appearance and will speed wound healing.

Three primary structural components of the dermis, collagen, elastin and GAGs have been the subjects of the majority of anti-aging research and efforts for aesthetic-anti-aging strategies pertaining to the skin, from "anti-wrinkle creams" to various filling agents. The primary aim of all skin anti-aging strategies is to reverse the dermal and epidermal signs of photoaging and chronological aging.

Chronic photodamage of the skin manifests itself as extrinsic skin aging (photoageing). DNA photodamage and UV-generated reactive oxygen species (ROS) are the initial molecular events that lead to most of the typical histological and clinical manifestations of chronic photodamage of the skin. Wrinkling and pigmentary changes are directly associated with premature photo-aging and are considered its most important cutaneous manifestations. The strategies aimed at preventing photo-aging include sun avoidance, sun protection using sunscreens to block or reduce skin exposure to UV radiation, retinoids in order to inhibit collagenase synthesis and to promote collagen production, and anti-oxidants, particularly in combination, to reduce and neutralize free radicals.

Healthy and normal functioning skin barrier is an important protector against dehydration, penetration of various microorganisms, allergens, irritants, reactive oxygen species and radiation. The skin barrier may be specifically adjusted to allow penetration of desired substances.

Regenerative medicine is the science of replacing, engineering or regenerating human cells, tissues or organs to restore or establish normal form and function. It uses cells, tissues, drugs, synthetic biomaterials and devices to help patients heal or regeneration.

## **Materials and methods**

The reason of different methods of skin regeneration is to increase skin regeneration, elasticity, smoothness, density, macroscopic and microscopic aspect, changing the skin condition. It is necessary to slow down ageing processes on a cellular level concomitantly. We can provide to the skin primary structural constituents, such as collagen, elastin, to prevent the formation of wrinkles, but some products and techniques do promote the natural synthesis of these substances except elastin enhancing. In the following, we will describe the methods that help us to obtain this kind of results.

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### ***Chemical peels***

A chemical peel involves the application of toxic chemical solutions to the skin in a controlled manner, producing controlled tissue death. The desired depth of the wound is dependent upon the condition to be treated. After the peel, the skin regenerates. The damaged skin likely regenerates through the growth of cells from deeper layers of the epidermis or from undamaged hair follicles.

Chemical peels are broadly defined by the depth of damage in the skin that they produce. They are categorized as superficial, medium, and deep. Superficial peels do not damage skin below the epidermis, the most superficial skin layer. Medium peels may reach to the superficial layer of the dermis, the deeper layer of the skin. Deep peels generally reach the deeper layers of the dermis. The depth of damage depends on the nature and concentration of the chemicals in the peeling solution and the length of time they are permitted to interact with the skin. Popular chemicals in peeling solutions include retinoids (tretinoin dissolved in propylene glycol), alpha-hydroxy acids (lactic acid and glycolic acid), beta-hydroxy acids (salicylic acid), trichloroacetic acid, and phenol (carbolic acid). Jessner's solution, a combination of resorcinol (14 g), salicylic acid (14 g), and lactic acid (85%) in ethanol (95%), is also an excellent peeling agent.

The indications for a chemical peel, since it is largely a cosmetic procedure, depend on the patient's tolerances and wishes for correcting skin textural problems. Treatments vary with the severity of the condition and the wishes of the patient. Indications for aesthetic concerns are as follows: photoaging, fine superficial wrinkling, dilated pores, superficial scars.

### ***Physical peeling***

Cryotherapy with liquid nitrogen or dry ice is recommended to obtain a tighter and a healthier skin, to increase metabolic and caloric burn, and also to produce collagen.

### ***Crystal-free microdermabrasion***

Microdermabrasion has remained a popular method for skin resurfacing and rejuvenation. This modality effectively exfoliates the outer layers of the epidermis using the propulsion of a mechanical medium, such as aluminum oxide microcrystals, at the skin through a handpiece. As the skin is exfoliated, the handpiece suctions the skin debris and microcrystals away through an accompanying vacuum. This modality has been used to treat light scarring, discolored and photodamaged skin, enlarged pores, and has been helpful in reducing the appearance of stretch marks and fine lines. This method also stimulates papillary dermis thickening. Despite its effectiveness, microdermabrasion is not recommended for all skin types and is used cautiously in persons with Fitzpatrick skin types IV–VI, rosacea, sensitive or thin skin because of the increased risk of irritation and hyperpigmentation. Though complications of microdermabrasion are minimal, patients' largest complaints post-procedure are temporary dryness and photosensitivity. Newer methods of microdermabrasion have moved toward microcrystal-free modalities exemplified by diamond tip microdermabrasion and hydradermabrasion.

The diamond tip microdermabrasion system is a handset with a contact point composed of diamond fragments that are adjustable for size and abrasiveness enabling treatment of a large variety of skin types and thicknesses. This method functions with a polishing motion to wear down the epidermal layers while the vacuum suctions away the debris and dead cells. Treatment is reliant on the operator's skill with the handpiece and the manual pressure applied to the epidermis along with the level of suction being used. Advantages of the diamond tip can be seen with treatments on areas around the eyes and mouth that can be treated without risk of microcrystals causing irritation, damage, or being ingested. Also, the tips are reusable reducing the cost of consumable microcrystals, with treatment times being shorter due to more effective clearance of debris and dead cells.

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The second developing method is hydradermabrasion. It works in the same manner as microdermabrasion but instead of exfoliating with propelled microcrystals, hydradermabrasion uses a combination of oxygen and aqueous solutions at supersonic speeds to remove debris and dead cells that are then suctioned away. This new modality is novel because as the treatment is exfoliating the epidermis, it is also hydrating the skin within the same treatment pass. This supersonic micro-droplet jet results in a pressured widening of micro-canals in the outer skin layers that facilitate greater hydration and cleansing of the skin. This moisturizing component allows for less irritation, reduced discomfort, and quicker recovery. Solutions and serums can also be formulated allowing better cleansing, extraction, and exfoliation while possessing antioxidant properties targeting specific skin types, textures, and conditions which cannot be delivered with traditional microdermabrasion. Hydradermabrasion has the same indications for use as microdermabrasion making it an excellent choice for persons with darker skin tones, aging skin, sensitive skin areas, oily, and dry skin complexions.

Further histological examination saw a replacement of elastic dermal tissue, collagen hyalinization, and fibroblast density correlating with a decrease in the appearance of fine lines, pore size, and hyperpigmentation in hydradermabrasion treated areas 6 weeks post-treatment with no patient complications. With its ability to add hydration, oxygen, and antioxidants to the exfoliated skin, this study demonstrates that hydradermabrasion treatment is effective at improving skin quality and should be considered an alternative to microdermabrasion.

### **LASER**

Nonablative skin rejuvenation or “subsurfacing” comes as a low risk and short downtime technology which can improve aging structural changes without disruption of cutaneous integrity. The mechanism of action is supposed to be a selective, heat induced denaturalization of dermal collagen that leads to subsequent reactive synthesis. Nonablative skin rejuvenation is not a precise term since rejuvenation is a controlled form of skin wounding aimed at achieving a more youthful appearance after the wound heals.

Treatment of photoaged skin has been divided into treatment of ectatic vessels and erythema, irregular pigmentation, and pilosebaceous changes and into the improvement of the dermal and subcutaneous senescence. The epidermis and superficial dermis can be selectively damaged by two basic mechanisms: (a) by targeting discrete chromophores in the dermis or at the dermal-epidermal junction or (b) by utilizing mid infrared (IR) lasers.

The devices for treatment of vascular and/or pigment irregularities include lasers emitting light at 532-, 585-, 595-, 755-, 800-, and 1064-nm wavelengths as well as filtered light generated by IPL systems equipped with different cut-off filters. Lasers emitting 1,320, 1,450, and 1,540 nm using interstitial and intracellular water as target chromophores and pulsed dye lasers (PDL) using oxyhemoglobin as the primary chromophore are now employed for Type II photo rejuvenation only. The clinical efficacy of these nonablative modalities are weaker than that of the ablative methods, however, new collagen formation and clinically observable improvement in wrinkles can be observed. Reduction of facial wrinkles by using IPL devices has shown less effect comparing to laser technology, but for type I photo rejuvenation, IPL systems have in general shown considerably better results than laser systems operating at subpurpuric energy levels. Ultrastructural and histological analysis confirmed effectiveness of absorption of light (532, 585, 595, with or without 1064-nm Nd:YAG laser) in the blood vessels of the superficial dermis, resulting in the release of inflammatory mediators and growth factors into the interstitium followed by stimulated fibroblast activity and initiation of tissue repair and enhanced collagen and elastin neoformation replacing the originally damaged elastic tissue. The increase in dermal collagen has also been confirmed by noninvasive ultrasonographic analysis and radioimmunoassay.

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Nonablative skin rejuvenation should not yet be considered an alternative for laser resurfacing. However there are interesting data showing comparative histological changes between the ablative and nonablative modalities.

Histological sections of skin before and after treatment with the different IPL devices have shown the formation of new collagen in the papillary and reticular dermis, as well as an increase in the number of fibroblasts and proportional decrease in the amount of solar elastosis is also usually found. If vascular and/or pigment disturbances improvement are immediate, the collagen remodeling response is delayed and maximum results are seen only between 3 and 12 months after treatment.

Laser resurfacing has been shown to be effective in counteracting photoaging through entire epidermal ablation, collagen shrinkage, stimulation of neocollagenesis, extensive dermal remodeling, regeneration of cellular organelles and intercellular attachments but parallelly, results in long recovery time are associated with risks of severe long lasting side effects, such as persistent erythema, hypo- or hyperpigmentation, infection or scarring.

Recently, fractionated CO<sub>2</sub>- erbium glass or erbium-YAG lasers have been introduced to reduce downtime and side effects. These devices emit light in a pixilated fashion onto the skin, producing an array of microthermal zones in the dermis. The controlled thermal stress to the epidermis and the dermal compartment is followed by a wound healing response ultimately leading to re-epithelialization and dermal remodeling.

Although the underlying molecular changes induced by different ablative and non-ablative as well as thermal and non-thermal skin rejuvenation treatments are not fully understood, there are investigations suggesting important roles of heat shock proteins (HSP), transforming growth factor  $\beta$  (TGF- $\beta$ ), different MMPs, synthethases, hyals and hyaluronic acid (HA). Type I and type III procollagen mRNA was also elevated for at least 6 months.

Monopolar RF is a noninvasive way to obtain skin tightening and immediate collagen contraction with a single treatment. Unlike lasers, the RF technology produces electric current, which generates heat through resistance in the dermis and as deep as the subcutaneous fat. Unfortunately there is a lack of long-term studies of efficacy and analysis of side effects for the skin using this method of skin rejuvenation.

It is obvious that different treatment modalities using visible light devices have resulted in varying clinical effects and allow to select individual treatment parameters for different indications. For this reason, careful simultaneous evaluation of any pigment disturbances, vascular abnormalities, wrinkles, and cutaneous sagging as skin layers are all linked is highly recommended.

### **Mesotherapy**

Mesotherapy, commonly known as “biorejuvenation” or “biorevitalization”, is a non-surgical method of correction of different aesthetic skin problems. The word “mesotherapy” comes from the Greek word “meso-“, which means “middle” and “therapeia” – to treat medically. In other words, this is a multiple injection introduction of different pharmaceutical, homeopathic or plant extracts, but also vitamins or microelements, in the dermato stimulate the biosynthetic ability of fibroblasts and facilitate interaction between cells and is intended to increase collagen and elastin production.

It appeared that mesotherapy could help for many aesthetic problems: it fights baldness, removes marks and scars, corrects cellulite, stops the process of skin-aging. It works successfully after plastic surgery intervention for faster recovery of the skin of the face and body, after peeling, after laser resurfacing by decreasing the recovery period. It can be wonderfully combined with introduction of botox, hyaluronic acid for wrinkles and all other non-surgical manipulations. By a series of mesotherapies the skin of the face becomes smooth, it tightens and revitalizes. It has new

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radiance and hydration. It is applied to face, décolleté, back side of hands and body. It takes 3 to 6 treatments, depending on the products introduced.

Different injection techniques can be used in mesotherapy: the intra-epidermal technique; the papular technique, in which reagents are injected into the dermo-epidermal junction; the nappage method, in which injections penetrate to a depth of 2–4 mm and are delivered at an angle of 30–60°; point-by-point injection into the deep dermis.

Mesotherapy is used to: remove fat in areas like the stomach, thighs, buttocks, hips, legs, arms, and face, reduce cellulite, fade wrinkles and lines, tighten loose skin, recontour the body, lighten pigmented skin, treat alopecia, a condition that causes hair loss. The technique uses very fine needles to deliver a series of injections into the middle layer of skin. The idea behind mesotherapy is that it corrects underlying issues like poor circulation and inflammation that cause skin damage.

#### ***'Vampire'/'Dracula' Lift (PRP – platelet rich plasma)***

Platelet Rich Plasma (PRP) therapy of the skin, also known as the ‘Vampire facelift’ or ‘Dracula facelift’ is a revolutionary regenerative aesthetic treatment that encourages the skin to build more collagen and elastin and thus renew and rejuvenate itself. The treatment involves taking a blood sample from the patient, from which we then isolate a certain fraction of the blood, which is particularly rich in platelets. After application of a numbing cream for pain relief, this fraction of the patient’s own blood is injected back into the skin using a special technique.

Platelets contain many beneficial growth factors. Upon reinjection the platelets release these growth factors, which trigger surrounding skin cells to proliferate and stimulate collagen production, thus helping to repair and regenerate damaged tissue.

Growth factors, including platelet-derived growth factor (PDGF), transforming growth factor (TGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF), are secreted from the  $\alpha$ -granules of concentrated platelets activated by aggregation inducers. These factors are known to regulate processes including cell migration, attachment, proliferation and differentiation, and promote extracellular matrix (ECM) accumulation by binding to specific cell surface receptors. It has been shown that PRP may induce the synthesis of collagen and other matrix components by stimulating the activation of fibroblasts, thus, rejuvenating the skin.

PRP therapy is a great addition or in some cases even alternative to other non-surgical cosmetic treatments such as ‘botox’ and fillers. It’s considered a very natural treatment, as it uses the patient’s own cells and growth factors to stimulate tissue repair, rather than injecting synthetic substances. The molecular mechanisms underlying PRP-inducing wound healing processes are still largely unknown and experimental studies confirming the effects of PRP on aged fibroblasts are very limited.

#### ***'Vampire'/'Dracula' PLUS (Advanced PRP)***

The ‘Vampire’/‘Dracula’ PLUS lift is a combination of platelet rich plasma (PRP) with infusion of a high-grade cell nourishing cocktail. In this advanced PRP treatment, it can be mix the patient’s own platelet rich plasma with a sterile solution containing more than 50 key skin optimization ingredients, before reinjecting it back into the skin. Apart from hyaluronic acid, the revitalizing solution used contains 12 vitamins, 23 amino acids, 6 coenzymes, 5 nucleic acid bases, 6 minerals and 1 special antioxidant, proven to protect and stimulate skin cells.

#### ***Dermal Fillers***

The goal of skin biorejuvenation is to increase the biosynthetic capacity of fibroblasts, inducing the reconstruction of an optimal physiologic environment, the enhancement of cell activity, hydration, and the synthesis of collagen, elastin and HA (hyaluronic acid). The desired effect could be achieved by the microinjections in the superficial dermis of products containing

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only one active ingredient or cocktails of different compounds which are perfectly biocompatible and totally absorbable: HA, vitamins, minerals, nutrients, hormones, GF, amino acids, autologous cultured fibroblasts, homeopathic products, etc. The distinct formulations can induce strikingly divergent molecular and cellular processes in fibroblasts *in vitro*. However, more detailed studies are required to elucidate whether and how the cellular and molecular processes are involved in facial skin rejuvenation *in vivo*, whether these processes are similarly efficient, independent of the age of the patients. The proof of concept, including long-term efficiency, optimal injecting protocols are still lacking too.

### ***Dermapen***

The Dermapen microneedling is a device that looks like a pen, that uses sterile, individually-sealed, disposable needle cartridges for optimal patient safety and comfort. Every Dermapen needle tip is actually made up of 12 smaller needles, which use proprietary technology to penetrate vertically into the skin. This creates small, micro-skin channels, while stimulating the skin's natural ability to heal itself and increasing the production of new collagen — which is the formation of strong, insoluble fibers that serve as connective tissue between cells.

These micro-skin channels carry up to 80% more topical nutrients, allowing penetration deep into the dermis fibroblasts, feeding the cells found in the dermis and basal layers. This increases the absorption of active ingredients into the skin, stimulating repair at a cellular level and accelerating the regeneration of the skin's epidermal cells. This treatment is similar to laser treatments, but without the severe side effects or the long recovery time. Adding to that benefit, studies have shown that Dermapen treatments are more effective than traditional microdermabrasion and chemical peels, due to how the disposable needles reduce the risk of cross-contamination. The adjustable needle depth also allows treatment that adapts to individual client's needs.

Microneedling being the advanced skincare treatment will reduce the appearance of wrinkles, fine lines, scarring, sun damage, improves the skin tone and texture, and give you plumper and smoother skin. As the treatment improves your skin's ability to self-repair by producing new collagen and elastin, you will see the exceptional results with minimal downtime. You will have the firmer skin and more youthful skin with this safe, chemical free treatment.

The microneedling procedure is easily tolerated by almost every patient and it can be tailored by going deeper in the specific areas where skin damage needs a stronger approach. When you start this treatment, after one or two treatments, you will see the reduced signs of the following: wrinkles and fine lines, scarring, enlarged pores, acne scars, stretch marks, skin sags, hair loss, burn scars, pigmentation marks from sun damage or acne, improve skin tone, stimulate the production of collagen and elastin.

Jett Plasma Medical it is method that use the result of the physical agents, like ions, electrons, neutrals, photons, nitric oxide, hydrogen peroxide on plasma. It is recommended for improving the skin color and structure, increasing its tone, elasticity, flexibility, for activation of the process of production of collagen fibers, which continues for 12 months after the procedure, and for smoothing age wrinkles of different depth. The result is a pronounced visual lifting effect and the observed reduction of the number of wrinkles of neck, legs, arms, hands, feet, including those around the lips and eyes. Jett Plasma Medical is a safe method because the superficial layers of the skin are not injured.

Botulinum toxin (BTX) has no effect on skin texture and cannot discontinue the skin aging process. However, regular BTX injections can slow down the visible aging process by helping in the management of certain dynamic facial lines and wrinkles. Current treatment options of exaggerated frown lines, glabellar lines or crow feet such as surgery or implants, do not address

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the underlying cause of these lines, namely the excessive nerve stimulation. The mechanism of action of BTX makes it an ideal agent to target the major cause of these dynamic lines.

### **Results and discussion**

The desired therapeutic anti-aging effect of the skin is a continuous, step-by step process, which combines various methods of the skin bio-revitalization and rejuvenation, augmentation, restoration of each skin layer individually and in the light of many other factors—from a style of the life to the immune, genetic, emotional and health status in general.

Simultaneously it is necessary to prevent wrinkle formation by reduction of inflammation (topical or systemic antioxidants) in combination with sunscreens and retinoids to enhance their protective effects. An important attention must be accorded to Transforming Growth Factor- $\beta$  family, other TGF- $\beta$ -based approaches (Decorin and Mannose 6 Phosphate), modulation of Smad3/Smad7 Signaling, Epidermal Growth Factor family, Fibroblast Growth Factor family, Platelet-Derived Growth Factor family, Granulocyte Macrophage-Colony Stimulating Factor, Connective Tissue Growth Factor, Interleukin 10, Connexins, other approaches under investigation for scar reduction, collagen, fibronectin, laminin, elastin, glycosaminoglycans and other natural biomaterials.

### **Conclusions**

Regenerative Aesthetic Dermatology is focused on innovative treatments to support the skin in restoring and regenerating old and/or damaged tissue and thus improving overall skin quality, promoting faster healing while minimizing downtime and side effects for patients. The onset of effects could be instantly after procedure or can be observed gradually increasing over the time (weeks or months), as we are determining the skin to help itself.

The regenerative treatments are encouraging the skin to build more collagen and elastin itself by bio-stimulation: resurfacing the epidermis (topical drug application, ablative LASERs, LASER radiofrequency resurfacing, ablative radiofrequency, microneedling, plasma skin resurfacing, crystal-free microdermabrasion), the formation of new collagen (IPL, LASERs, radiofrequency, infrared, Jett Plasma Medical, medical needling, mesotherapy, platelet-rich plasma, fillers as hyaluronic acid, *HydraFacial MD*, *Oxygen facial*, *chemical peels*, hormone replacement therapy, autologous and allogeneic stem cells, gene therapy), slow down the visible aging process by helping in the management of certain dynamic facial lines and wrinkles (Botulinum toxin).

While natural aging is genetically determined, extrinsic aging can be prevented. Aesthetic dermatology should contribute to “healthy aging” not only in cosmetic means by trying to erase time vestiges in skin but by also playing a significant part in prevention, regeneration, and delaying of skin aging combining knowledge of possible local and systemic therapy, instrumental devices and invasive procedures, filling the lack of scientific investigations and becoming one of the important focuses of the aging research.

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## Oxidative stress, inflammation and endothelial dysfunction: implications in atherosclerosis. Note II.

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### Abstract

NADPH oxidase (*nicotinamide adenine dinucleotide phosphate-oxidase*), with its generically termed NOX isoforms, is the major source of ROS (*reactive oxygen species*) in biological systems. The oxidant/antioxidant imbalance in favor of oxidants in the vascular system generates ROS via activation of NADPH oxidase. ROS are small oxygen-derived molecules with an important role in various biological processes (physiological or pathological). Some processes are beneficial and necessary for life under physiological conditions, but they are noxious, harmful under pathophysiological conditions. NADPH oxidases are present in phagocytes and in a wide variety of nonphagocytic cells. The enzyme generates superoxide by transferring electrons from NADPH inside the cell across the membrane and coupling them to molecular oxygen to produce superoxide anion, a reactive free-radical. Structurally, NADPH oxidase is a multicomponent enzyme which includes two integral membrane proteins, glycoprotein gp91<sup>phox</sup> and adaptor protein p22<sup>phox</sup>, which together form the heterodimeric flavocytochrome b558 that constitutes the core of the enzyme. During the resting state, the multidomain regulatory subunits p40<sup>phox</sup>, p47<sup>phox</sup>, p67<sup>phox</sup> are located in the cytosol organized as a complex. The activation of phagocytic NADPH oxidase occurs through a complex series of protein interactions. The increased production of free radicals under pathophysiological conditions is an integral part of the production of cardiovascular diseases and in particular of atherosclerosis. At the onset and progression of atherosclerosis, various non-traditional intercorrelated risk factors contribute such as oxidative stress, inflammation and endothelial dysfunction. Oxidative stress plays a crucial role not only in the formation but also in the evolution and destabilization of lesions. Oxidative stress is closely linked to endothelial damage. Endothelium modulates vascular tone by releasing specific vasoactive substances. At the onset and progression of atherosclerosis contributes to decreasing the bioavailability of NO (nitric oxide) or EDRF (endothelium-derived relaxing factor) with an important role in conserving vasodilation and inhibiting vasoconstriction. Clinical and paraclinical investigations show that inflammatory reactions operate at all stages of atherosclerotic events. According to the theory of oxidative stress, atherosclerosis is also the result of the particularly oxidative changes of LDL (low density lipoproteins) in the arterial wall. Excess ROS can produce peroxinitrite with NO, the cytotoxic oxidant important mediator of LDL oxidation with proatherogenic action.

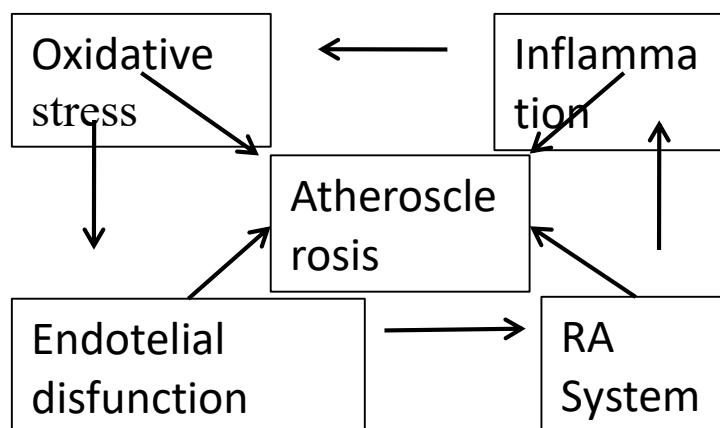
**Keywords:** EDRF, NADPH Oxidase, NOX, Reactive Oxygen Species.

### Introduction

Reactive oxygen species, conventionally seen as harmful end-products resulting from aerobic metabolism, have received special attention due to multiple cellular sources, cellular effects, but especially the consequences that produce them when they are produced in excess.

Among the many sources of ROS in biological systems, including the vascular system, the family of redox enzymes - NADPH oxidases (NOXs) [1]. Reactive oxygen species (ROS) were considered as phagocytic origin initially. Their function as body defense (antimicrobin, antifungal, antibacterial) has been shown. Subsequent research has shown that similar NADPH oxidases (isoforms) have also been described in a wide variety of non-phagocytic (somatic) cells that slowly and sustained generate intracellular  $O_2^-$  in different compartments (intracellular, extracellular). [1, 2, 3] The production of ROS ( $O_2^-$ ,  $H_2O_2$ ) by NADPH oxidase is achieved through the catalytic subunit of NOX which differentiates it from other oxidases. [4] Although all NOXs isoforms have the same primary function in generating ROS of whatever origin, they differ from several points of view: structural, functional, regulatory factors, component subunit requirements, cellular and subcellular distribution, as well as contributing to the development various pathological conditions, including cardiovascular diseases. [1, 3, 5, 6, 7, 8, 9] While phagocytic NADPH oxidases become active after stimulation to produce superoxide radical anion, NADPH somatic oxides by the catalytic subunit are continuously active as a slow and sustained intracellular source of  $O_2$ . ROSs resulting from the NADPH oxidase family members activity by the NOX subunit act as intracellular signaling molecules, important regulators of key biological activities such as cell growth, proliferation, differentiation, migration, apoptosis.[10]

Produced in excess (condition of oxidative stress) ROS reacts indiscriminately. They may cause irreversible alterations in most biological molecules which also affects important cellular functions contributing to a wide variety of conditions such as inflammation, endothelial dysfunction, cardiovascular disease, hypertension, diabetes, chronic kidney disease, cardiac hypertrophy, stroke etc. [11, 12] There is a complex pathophysiological process in the development of atherosclerosis involving oxidative stress, inflammation, endothelial dysfunction, NO production, etc. In this context, the paper presents recent data from the literature on the oxidative stress triad, endothelial dysfunction, inflammation that serves as a basis for the development and evolution of the multistage process - atherosclerosis, schematized by Husein et al. (Figure 1) [13]



**Fig. 1:** The relationship between oxidative stress, inflammation, endothelial dysfunction, atherosclerosis, renin-angiotensin (RA)

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### ***Oxidative stress in the pathogenesis of atherogenesis***

In recent years, a huge amount of data has accumulated that support the direct link between excess free radicals (ROS) that causes oxidative stress (redox status) in different compartments (intracellular, extracellular) and most known diseases, including atherosclerosis. In this regard, it is proven by numerous experimental and clinical studies that oxidative stress is closely linked to various risk factors for atherosclerosis, such as hypercholesterolemia, hypertension, diabetes, smoking.

The importance of ROS in vascular physiology and pathology under oxidative stress conditions is obviously increasing because several vascular sources (endothelial cells, vascular smooth muscle cells, fibroblasts), infiltrating immune cells contribute to the production of ROS mainly via NADPH oxidase non-phagocytic. [14] Both basal and stimulated NOX activity occurs in all segments of the vascular wall (intimate, media, adventitia), where these immune cells can reach.

Into the vascular system are expressed the major isoforms of NADPH oxidase - NOX1, NOX2, NOX4 and NOX5 that differ by distribution, different mechanisms of activation, response to a wide variety of heterogeneous factors (physical, chemical, environmental or biological) and by potential contribution to vascular disease [15, 16], so that NOX as ROS in the vascular system can have both beneficial effects that in the context of physiological conditions maintain major vascular processes as well as harmful effects under overproduction conditions of reactive species derived from oxygen. [16] Oxidative stress caused by risk factors is a major cause of endothelial dysfunction.

### ***Endothelial dysfunction and oxidative stress***

The endothelium plays a critical role in regulating vascular function by releasing paracrine factors that maintain vascular tone, inhibits platelets and adhesion of inflammatory cells, promotes fibrinolysis, and limits vascular proliferation. Vasodilatation is mainly mediated by factors such as NO or relaxation factor derived from endothelium derived-relaxing factor (EDRF) and prostacyclin while vasoconstriction is mediated by factors such as endothelin-1, angiotensin II, thromboxane A2, prostaglandin H2. [17, 18, 19]

Endothelial dysfunction (vascular endothelium) is characterized by modifying the properties of endothelial vasodilation consisting of the reduced pro-inflammatory state, prothrombotic status. Endothelium is a selective and metabolically active barrier that plays a crucial role in regulating vascular homeostasis by maintaining a delicate balance between vasodilatation and vasoconstriction. [17, 18]

Endothelial dysfunction, oxidative stress, inflammation and dyslipidemia play a vital and vital role in the development and progression of atherosclerotic lesions. Under physiological conditions, endothelium controls vascular inflammation by releasing NO. Endothelial dysfunction can be regarded as a primum movens of atherosclerotic disease characterized mainly by endothelial overproduction of ROS (as O<sub>2</sub>) to lower bioavailability of the NO precursor recognized as the start of atherosclerosis and increase reverse angiotensin II in the vascular injury is caused by inducing the generation of oxidative species by activating NADPH oxidase. [12]

Endothelial function is impaired in the early stages of atherogenesis, and is closely related to the atherosclerotic risk factors. [19] NO production is crucial for maintaining normal endothelial vascular integrity. NO as a vasodilator at physiological (nanomolar) concentrations mediates endothelial protective functions by inhibiting neutrophil activation and adhesion, adhesion and platelet aggregation, vascular smooth muscle proliferation, proinflammatory cytokine expression, prevention of development and complications of atherosclerosis. Numerous experimental and clinical studies have shown that oxidative stress is closely linked to various atherosclerosis risk

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factors. Oxidative stress caused by risk factors is the major cause of endothelial dysfunction as a common condition predisposing to atherosclerosis. Endothelial dysfunction predisposes to atherosclerotic lesions and has been proposed as an important diagnostic and prognostic factor for coronary syndromes. Endothelium and its major NO product are key regulators of vascular function. The pathogenesis of endothelial dysfunction is multifactorial in which oxidative stress appears to be the common cellular mechanism. [18] Increased production of ROS reduces the production and consequently the bioavailability of NO that results in vasoconstriction, platelet aggregation, and adhesion of neutrophils to the endothelium. Also, the interaction of O<sup>2-</sup> with NO leads to peroxynitrite, a less effective substance for activating guanylyl cyclase, and thus the bioavailability of NO becomes particularly low.

#### ***NADPH oxidize in the regulation of vascular inflammation***

Inflammation and oxidation are two closely related basic processes, which support the pathogenesis of most disease states in humans. These two distinct mechanisms (inflammation and oxidation) are constantly reciprocal, with obvious interactions in the vascular wall. [16, 20] Both processes are simultaneously encountered in many pathological conditions [21, 22] including cardiovascular diseases. [23] Traditionally, vascular inflammation begins with activation of the arterial wall endothelium where VCAM-1, ICAM-1 adhesion molecules that express monocytes are expressed, and then migrate through the endothelial layer under the influence of various proinflammatory chemoattractants. [24]

Inflammatory reactions induce ROS production, but the reverse is equally true. Moreover, components of the reaction interact with synergistic effect. Inflammatory reactions are caused by endogenous and exogenous aggressions and are characterized by cellular and vascular events. Inflammation and oxidation are processes involved in the pathogenesis of most human disease states. These distinct mechanisms are reciprocal and have obvious interactions in the vascular wall. [25] Traditionally it is considered that vascular inflammation is initiated at the luminal surface, the layer progresses through the media adventitial. [26] There is evidence suggesting that vascular adventitia is first activated in a variety of cardiovascular diseases and has key role in shaping and evolution of vascular inflammation. [27] Vascular inflammation is part of the defense and tissue repair process and is also involved in many pathological conditions such as cardiovascular disease. Traditionally vascular inflammation begins with endothelial activation and leukocyte extravasation followed by the inflammatory response that spreads from the blood vessels to the environment. [28] The production of ROS by NADPH oxidase is achieved via NOX, which differentiates NADPH oxidase from other oxidases. Increased production of ROS after induction or activation of NADPH oxidase in response to cardiovascular risk factors and inflammation contributes to the development of endothelial dysfunction and cardiovascular disease. Vascular endothelium plays an important role in regulating the inflammatory response after trauma or haemorrhage. [28]

Vascular inflammation is implicated in both local and systemic inflammatory conditions. Endothelial activation and leukocyte extravasation are key events in vascular inflammation. [29] Inflammation, considered as a primary process, plays a fundamental role in all stages of atherosclerosis from initiation, evolution, to thrombotic complications.

There are numerous studies showing that cardiovascular disease (and not only) have an important inflammatory component involved in triggering the disease in atheromatous plaque formation, development and worsening. In other words, chronic inflammation in the body, especially in the cardiovascular system, plays a major role in the development and worsening of atherosclerosis and other cardiovascular diseases.

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### ***NOX and atherosclerosis***

Pathogenesis of cardiovascular disease is one of the most complex human diseases. A large number of risk factors, physicochemical interactions, cell types, involved biological processes add to the complexity of these diseases, including atherosclerosis. Many arguments prove the role of oxidative stress and inflammation in promoting atherosclerotic cardiovascular disease. [30]

Atherosclerosis is a multifactorial disorder that takes place in several stages and involves large and medium arteries. Preclinical and clinical investigations show that inflammation operates in the pathogenesis of each stage of atherosclerotic events (onset, progression, complexity of lesions). It is recognized that vascular oxidative stress as a key factor for the initiation and evolution of atherogenesis contributes to the destruction of endothelial cell homeostasis [12, 31, 32, 33], which affects the balance between vasoconstriction and vasodilation [12] and initiates a cascade of inflammatory processes both by promoting infiltration of inflammatory cells into the wall both vascularly and indirectly through the induction of cytokines and other inflammatory mediators that eventually lead to structural and functional manifestations of the disease.

Traditionally, vascular inflammation begins with endothelial activation and leukocyte extravasation and ultimately resulting in an inflammatory response that spreads from the blood vessels to the environment. NADPH oxidase, the major source of ROS in blood vessel cells, is an important factor for the onset and development of vascular disease through endothelial dysfunction, inflammation etc. [34]

Thus, inappropriate activation may contribute to the development of cardiovascular diseases such as hypertension, atherosclerosis, diabetes, cardiac hypertrophy, heart failure, ischemia-infusion, stroke etc. [35, 36, 37, 38, 39]

### **Conclusions**

Oxidative changes in the arterial wall may contribute to atherosclerosis when the balance between oxidants and antioxidants changes in favor of the increasing of the first. Vascular endothelium also produces paracrine factors that maintain vascular homeostasis; one of these factors is NO. [40]

The link between endothelium-derived NO and vascular health is likely to be due to the pleiotropic effects of NO on the vascular wall. Common to these processes is the increased bioavailability of ROS, low levels of NO (important vasodilator with protective physiological role in the vessels, it is crucial for maintenance of vascular endothelium health and function) and reduced antioxidant capacity in cardiovascular, renal, nervous systems, etc. The NO bioavailability depends on the processes that control NO synthesis and degradation as well as the sensitivity of the target tissue to NO. [41]

Continuously synthesized endothelium has a wide variety of biological properties that maintain vascular homeostasis including modulating vascular dilatation tonus, regulating local cell growth and protecting vessels from injuries of circulating platelets and cells and multiple antiatherosclerotic properties. [27]

High concentrations of ROS increase premature degradation of endothelial-derived NO and NADP production, resulting in endothelial dysfunction. [22] Blocking of NO synthesis with NOS inhibitors results in significant peripheral vasoconstriction and increased blood pressure. In addition to blood pressure control, NO protects vessels thrombosis by inhibiting platelet aggregation and adhesion.

The initial events in atherogenesis is considered increased transcytosis of low-density lipoproteins and their subsequent storage, retention and modification of the endothelium. [27]

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It is followed by the infiltration of activated inflammatory cells from the coronary circulation into the arterial wall. Here they secrete ROS and produce oxidized lipids capable of inducing apoptosis of endothelial cells. It is a chronic inflammatory response in the arterial wall, mainly by promoting oxidative alteration and other types of LDL alteration [27, 36, 42], changes known as atherogenic, and at the same time is one of the early events of atherosclerosis.

Modified lipoproteins are proinflammatory as they signal the endothelial expression of leukocyte adhesion molecules and chemokines. In addition, direct and indirect evidence supports in particular the contribution of Nox 1, Nox 2 to atherosclerosis. [42, 43] ROS, in particular O<sup>2-</sup>, is involved in the pathogenesis of each stage of vascular lesion formation in atherosclerosis.

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