ISSN: 1220-3173; E-ISSN: 2457-7618

THE DETERMINING ROLE OF THE ACROSOME REACTION IN FERRET'S *(MUSTELA PUTORIUS FURO)* FECUNDATION

ROLUL DETERMINANT AL REACȚIEI ACROZOMIALE ÎN REALIZAREA FECUNDITĂȚII LA DIHOR *(MUSTELA PUTORIUS FURO)*

N. PĂSTÂRNAC[1)], G. PUCHIANU[2),*)], A. COMAN[3)], I. COMAN[2)]

ABSTRACT | REZUMAT

The performance and the completion of this family of *Mustelidae's* fecundity are mainly determined by the acrosome reaction. In order to visualise the amphimixis process of the two opposed genders gametes in ferrets, the stereomicroscopic and the microscopic examination of the fluid resulting from the lavage of the uterine horns and oviducts were performed. To achieve this, in 20 days following the mating cycle (March–April), experimental sacrifices were performed in three categories of ferrets: adult females, young females, females suffering from various illnesses of the genitalia and females which mated and died. The samplings for analysis purposes were performed in the period between March and April, when most of the blastocysts elongate or attempt implantation. In order to harvest all the elements existing in the analysed fluid (mainly the zygotes), the examination was performed by droplet (by micropipetting), which although is relatively more difficult to perform and needs more work time, has the advantage that it offers an impeccable accuracy and exactness. It is useful to mention that this particular genus is the only one in the *Mustelidae* family which has the second cycle of heats and mating between July and August. In fertilized eggs can be noticed the penetration process of the spermatozoon's head through the external egg membranes, having crossed this barrier, a process which is mitigated by certain acrosome enzymes, with the help of which it 'digs' a tunnel through the protoplasmic mass which facilitates the spermatozoon's nucleus to reach the ovule's nucleus envisaging the amphimixis, by the mutual resorption of the two haploid nuclei and finally towards the creation of a new, unique diploid formation, that of the future embryo. This vital process is based on the determining role of the acrosome reaction, an indispensable link of mammals' reproduction embryogenesis. The chances to actually capture this process are very slim, benefitting in this chance to catch the moment of the amphimixis in our experience with the minx.

Keywords: zygote, ovule, acrosome reaction, fecundity

Realizarea și finalizarea fecundității acestui mustelid este determinată în primul rând de reacția acrozomială. Pentru a surprinde procesul de amfimixie a celor doi gameți de sex opus la dihor, s-a procedat la examinarea stereomicroscopică și microscopică a lichidului provenit din efectuarea lavajului coarnelor uterine și oviductelor. Pentru aceasta la un interval de 20 de zile de la realizarea montei (martie–aprilie) a primului ciclu de montă s-au efectuat sacrificări cu scop experimental la trei categorii de dihori: femele adulte, femele tinere, femele cu diferite afecțiuni ale aparatului genital și femele montate și moarte. Recoltările pentru analiză s-au efectuat în perioada martie - aprile, când majoritatea blastocițtilor se alungesc sau încearcă să se nideze. Pentru a putea recolta toate elementele existente în lichidul analizat (în primul rând zigoții), s-a procedat la efectuarea examenului în picătură (prin micropipetare), care deși este relativ mai dificil de efectuat și presupune un timp mai îndelungat de lucru, are avantajul faptului că oferă exactitate și fidelitate ireproșabilă. Este util de menționat că acest mustelid este unicul care are al doilea ciclu de călduri și montă iulie – august. Din ovulele fecundate a fost surprins procesul de penetrare a membranelor ovulare externe de către capul spermatozoidului care a trecut de această barieră, proces care se realizează cu ajutorul unor enzime acrozomiale cu ajutorul cărora „sapă” un tunel prin masa protoplasmatică care facilitează nucleul spermatozoidal să ajungă la nucleul ovular în vederea amfimixiei acestora și prin resorbția reciprocă a celor doi nuclei haploizi, și în final la crearea unei formațiuni unice diploide, cea a viitorului embrion. Acest proces vital se bazează pe rolul determinant al reacției acrozomiale, verigă indispensabilă a embriogenezei reproducției mamiferelor. Probabilitatea surprinderii acestui proces, este mică, noi beneficiind de această șansă a surprinderii momentului de amfimixie din experiența de la nurcă.

Cuvinte cheie: zigot, ovule, reacție acrosomială, fecunditate

The fecundity of a headcount of ferrets can be affected by various illnesses of the reproductive system and can be considered partially and, in some cases, to be totally compromised. In certain sick ani-

1) AGMVR Brașov, Romania
2) Transilvania University, Brașov, Romania
3) CMVI Hoghiz, Brașov, Romania
*) *Corresponding author: gpuchianu@yahoo.com*

mals, following the anoestrus, the heats resume and they occur apparently in a habitual fashion (1, 2, 5, 7).

The histomorphological analysis of the mating products in the female genital tract proves that the persistence of infertility and the decrease of fertility in ferrets is triggered by disorders of the ovogenesis and growth process, where the zygote (namely, the fertilized egg) is negatively influenced by the estrogen hormone when it is in its pre-segmentation period. The embryo develops by segmentation (division) (3, 4, 10, 11).

Estrogens are the hormones which produce changes in certain mammals' genital tract, including in Mustelidae, during their estral cycle, even during the follicular phase. One of the estrogen substances is estradiol (the ovarian hormone), one of its derivates being estrone or folliculin; another derivate is estriol, an estrogen present in the urine of vertebrate females and in the placenta during gestation, and in small quantities even in the adrenal glands and in the testicles (6, 13, 14).

In females, the estrogen hormones influence the development of primary and secondary sexual features, the seasonal occurrence of the estrus, the development of their mammals, stimulate the uterine contractions, intervene in the skin's and annexes' trophicity, etc. (15, 17).

The estrogens are steroid hormones including estriol, estrone and estradiol, which control the sexual development in ferrets (16).

MATERIALS AND METHODS

The success in capturing the amphimixis of the two opposed genders' gametes in minx has stimulated us in the attempt to perform a comparison with the ferret, as they are two rather similar species from a phylogenetic point of view, showing many of the same features (12). Just as in minx, the researches in ferrets were performed by random sacrifices, respectively in 20 days following the mating process of an experiential batch of 25 adult females, a batch of 18 young females and a batch of 18 females showing various illnesses of the genitalia.

The genitalia have been sampled in the period between March and May, being initially examined macroscopically, and then prepared for the performance of the uterine lavage, with the goal of totally recovering the blastocysts at this level. Following a prior examination of the ovaries using a binocular magnifying glass (magnifying power 2.5x25), we succeeded in collecting the entire quantity of fluid resulting from the lavage of both uterine horns and uterus. Using the stereomicroscope, the entire set of ovules has been examined, with the goal of capturing the amphimixis of the two opposed genders gametes, a process which takes place rather quickly, that being why capturing it was a very difficult task, mostly counting on chance (12). To this end, we used medical imaging and optic microscopy to examine the cells and sub-cells, the zygotes (fertilized eggs before segmentation, containing both female and male pronuclei). Following the sampling from a batch of 8 females coming from the casualties in March and April, we succeeded to compare these zygotes in various stages and of various ages to those harvested from the experimental batches.

The establishment of the performance of the fertilization process was performed following the examination of the number and development stage of the zygotes, using the microscope, as well as the lavage of the oviducts and the uterine horns. To this end we used, as in the case of the minx, the Paraipan method in order to harvest the material necessary for the examination (8, 9).

Following the detachment of the mesosalpinx, of the tubo-ovarian ligament and of the mesometer from the oviduct's body and from the corneal extremity, we performed two transversal sections, a superior one, in the vicinity of the utero-tubar junction, and an inferior one, in front of the cervical ostium. The perfusion technique is performed by introducing a thin, blunt needle in the uterine horn lumen, from the cervical ostium and towards the oviduct, the needle being connected to a 2 ml syringe filled with the perfusion fluid. Then the free extremity of the uterine horn is suspended above a watch glass, or in the lumen of a glass funnel, which is introduced in a sampling test tube.

This harvesting technique needs a cautious actuation of the syringe's piston, in order to avoid causing any damage to the blastocysts and in order to facilitate the gradual penetration of the perfusion fluid in the uterine horn. We mention that after we have inoculated one millilitre of perfusion fluid, the syringe was removed, was loaded with air, and then it was reconnected to the needle which was still in the corneal lumen, and air was pumped in. This has the role of pumping out the leftover fluid in the lumen, and then this operation is repeated, in the same manner.

As perfusion fluid, we used saline solution and Hank's salt solution. Where it is not necessary that the blastocysts to be kept alive, instead of the aforementioned perfusion fluids can be used 2% formaldehyde solution.

Depending on the purpose envisaged, each test tube must contain all the blastocysts in an uterine horn, its label mentioning the ferret's ID number, the horn from which the sample was harvested, as well as the number of dead and alive blastocysts, and data regarding the female's mating or death.

It is worth mentioning that the analysis sampling must be performed by April 20th, when most of the blastocysts elongate or attempt implantation. For the

microscopic examination of the components incorporated in the fluid resulted from the lavage of the uterine horns (gynogametes, zygotes in various stages of evolution, live or dead cells, etc.), several methods can be used: direct examination, examination of the blastocysts, examination of the histological sections, '*in toto*' examination, etc., in the present case using the first two of these methods.

The direct examination was performed using a stereomicroscope and a binocular magnifying glass, on the microscope's bracket placing the watch glass containing the Hanks perfusion fluid.

After the fluid has settled, the zygotes, being heavier, begin to sediment on the bottom and in the centre of the watch glass, and they are large enough to be observed with the naked eye.

In order to harvest all the elements existing in the analysed fluid (mainly the zygotes), the examination was performed by droplet (by micropipetting), which although is relatively more difficult to perform and needs more work time, has the advantage that it offers an impeccable accuracy and exactness.

RESULTS AND DISCUSSIONS

The examination of the blastocysts has revealed that they look like clear, unequal vesicles, even when their age is the same. Except these vesicular elements, we also noticed the presence of other smaller, more opaque formations, representing the blastocysts that died prior to the harvesting and which can be considered as 'blastocystic sand' (8).

Depending on the purpose envisaged, the complete blastocysts may be coloured using Methylene blue, and those crushed between the slides, using the Giemsa method.

Our approach regarding the fecundity process consisted in examining and going through the entire chain of possible logical events from which it can occur. The most difficult link in this chain has been to photographically capture the moment of amphimixis of the two gametes of opposed genders, from inside the protoplasmic mass, when the two nuclei – the female ovule and the male spermatozoon become one, following the joining and the mutual resorption of the two opposed gender gametes.

From the mating of the European Ferret (*Mustela putorius putorius*) with 2n=40 chromosomes with the Steppe Polecat (*Mustela putorius eversmanii*), with 2n=38 chromosomes, we can obtain viable offspring, confirming the fact that they are two mutations of the same species.

It is extremely important to mention that the different environmental conditions in individuals with the same genome will subsequently show various phenotypes.

The probability to capture this junction process is very low, by the extreme accuracy of our work and already having excellent results in the case of the minx, and also the strike of luck we had, we succeeded in capturing the amphimixis process, a decisive stage of the reproduction process. It begins when the sperm cells make contact with the ovule and in the end, the penetration of a single spermatic head and neck which has crossed the endoplasm, crashed into and has coupled with the ovule's nucleus (Fig. 3)

The penetration of the external ovular membranes, through which the head of the spermatozoon crosses this barrier, takes place with the help of an acrosomal enzyme, with the help of which it 'digs' a tunnel in the protoplasmic mass, which facilitates that the spermatozoid nucleus reaches the ovule's nucleus for their amphimixis and, by mutual resorption of the two haploid nuclei, the creation of a single diploid formation, that of the future embryo.

The result, known as the zygote, is nothing else than the fertilized egg before its segmentation, which has both opposed genders' pronuclei. It is worth mentioning that outside it, in the microscopic field can also be found blastocysts in various stages, blastocytic sand, as well as various cells which can be found in the cavity of the reproductive system's tract.

Therefore, the zygote, in its formation, begins with the contact and joining of the genders and with the mutual resorption of the two opposed genders gametes and the fusion of the pronuclei (the maternal and the paternal chromosomes). This moment is the first stage before segmentation (division by mitosis) of the zygote.

A more concise and explicit characterization of the two bivalent in this metaphase consists in the fact that they are formed from the bi-chromatid chromosomes which affix with their centromere on the filament of the spindle fibres and will travel towards the metaphysical pole, located at the cell's Equator and which are normally oriented in a bipolar manner on the fibres of that respective spindle. The centromeres of the homologous chromosomes randomly orient themselves on both sides of the Equatorial plane.

These events do not result only from the joining of the two opposed genders' nuclei in the ferret, but they are also the consequence of the egg fertilization by the spermatozoon, because in actuality this process is properly controlled and coordinated by the intervention of a series of very complex cellular and sub-cellular processes, in order to finally reach the fusion of the two parts. They evolve without any morphopysiological deviations, at the level of each functioning plateau, in order to achieve fertilization.

The contact ovular membrane has the role to prevent the penetration of any additional spermatozoa of the same male, or of other males, in case of hetero-

spermia. We would like to mention that the penetration in the ovule of the head and neck of the randomly selected spermatozoon is induced by the acrosomal enzymes, which subsequently facilitates the fecundation by triggering, stimulating and performing meiosis, in the same time with the formation of the second polar body (polocyte). The fusion of the female and male pronuclei makes the zygote to start segmenting and dividing by cleavage.

During the first stage, when the spermatozoa come into contact with the ovules, in many species and it seems that in ferrets as well, the spermatozoa' acrosome has the role of digesting a rich area of columnar follicular cells, surrounding the ovule's pellucid area, which persists a certain time after ovulation.

Following this aggressive action of the denudation acrosome of the ovule in the area rich in columnar follicular cells, surrounding the ovule's pellucid area like a halo, in the same time happens an attempt to approach the ovule's membrane, in order to pierce it and to penetrate inside it. This penetration process of the spermatozoon in the ovule's ovoplasm can be classified as the first stage of the acrosomal reaction, perfectly separate and visible outside the ovule and sometimes can also be noticed the attempt of the spermatozoon's head to penetrate the ovule's membrane structures, to digest the membrane of the envisaged ovule, which has facilitated the spermatozoon's fecundation.

The time necessary for the tail to detach, the massive release of enzymes, is enough for the head and neck to meticulously 'dig' through the ovulo-protoplasmic mass a well-defined tunnel, in order to cross the distance from its point of entrance and until its tangential approach of the ovule's nucleus.

If this stage would not take place in an extremely restricted plane and within a relatively short period of time, the aforementioned aspects would have been more easily noticeable microscopically, this being the reason for which we used a larger number of animals to perform our experiment and our strictness in the samples' harvesting.

In figures 1, 2 and 3 are presented chronologically the most important stages of ovules' fecundation in *Mustelidae*.

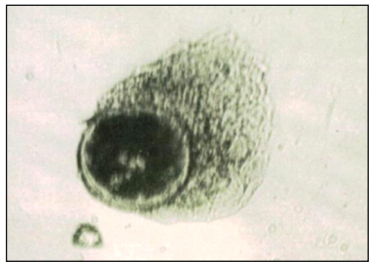

Fig. 1. The ovule, following the descent of the De Graff follicle, immediately after expulsion

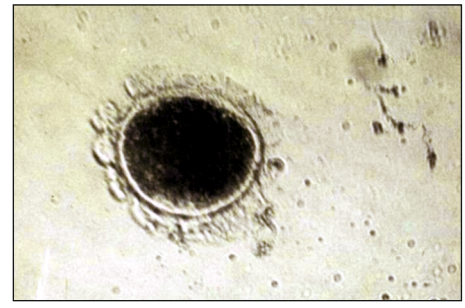

Fig. 2. Ovule, during full enzymatic assault phase, which represents the first stage of the acrosomal reaction (gradual denudation of columnar follicular cells)

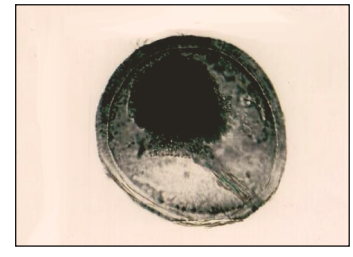

Fig. 3. One of the spermatozoa succeeds in penetrating the ovule, enzymatically making its adduction tunnel towards the ovule's nucleus, thusly performing the joining of the two haploid gender gametes into a diploid one

The penetration of the head and its circulation through the ovoplasmatic tunnel, on a trajectory slightly curved from left to right, for the travel of the spermatozoon's head towards the female nucleus, respectively towards the coupling area with the female gamete. This marks the beginning of embryogenesis.

If this process would not be a quick one, the evolution could not be a smooth one, and the synergic interaction would not be possible. As such, the evolution towards the performance of the fecundation process takes place harmoniously. We mention that the cells produced by the zygote's cleavage, named blastomeres, include the early ages of embryo's formation and until the formation of the blastocyst. The blastomeres suffer repeated divisions, without increasing their volume, as can be seen in Figure 4, even with the intention to involve in their dimension.

If by forming of the blastomeres, following the zygote's cleavage, for the formation of the blastocyst, dissolution is triggered during stagnation, until the total disappearance of the adduction tunnel of the male gamete towards the ovule's nucleus.

That process is a vital one, because it conditions the synapse, or the joining of the homologous chromosomes in meiosis, merger of the nuclei, the karyogamy and in the end, the fusion of the gametes in the fecundation process.

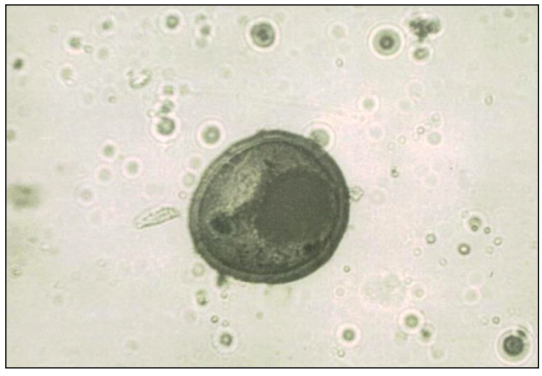

Fig. 4. When the spermatozoon's head, represented 90% by its nucleus, begins the mutual resorption with the female nucleus. Its transit tunnel through the ovular protoplasma begins to close, starting from its point of entry through the ovular membrane and will close in the junction point of the two opposed genders' nuclei.

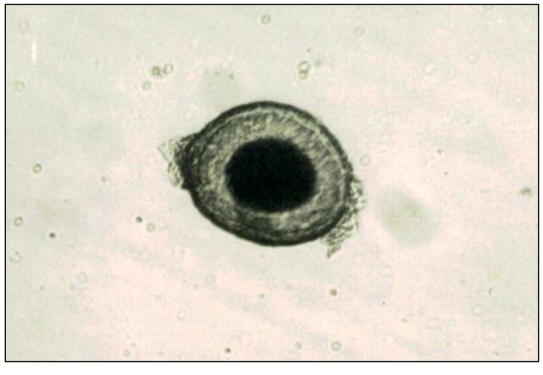

Fig. 5. Cleavage, or the division of the zygote in blastomeres, is holoblastic, respectively awaiting nidation or fixation by implantation in the uterine mucosa of the female ferret.

In the performance and completion of the fecundation in these Mustelidae, the determining role is played by the acrosomal reaction. The lack or the disruption of this reaction unavoidably leads to sterility.

All the concomitant processes happening in the sub-cellular structures, during the entire fecundation process, have an important role due to the acrosome, which possesses a vesicle containing enzymatic and hydrolytic enzymes (especially hyaluronidase and acrosin), which facilitates the penetration of the outer layer of the ovule's membrane, in order to penetrate into the ovule's cytoplasm.

It is difficult to pinpoint when and where these selective receptors allow the penetration of a single spermatozoon into the cytoplasm, in order to trigger in the ovule of the lysosomal reaction, by creating a transit duct for the spermatozoon's head and neck.

Lysosomes are cellular organelles in the ovular cytoplasm, with a morphologic structure and of various sizes, respectively between 0.05–0.5 mm, encapsulated in a glycoproteic sheath or membrane, inside which can be found about 40 various hydrolytic enzymes (phosphatases, acid hydrolases, proteases, nucleases, polysaccharides, lipases, etc.). In conclusion, these lysosomes are digestion cellular organelles of certain macromolecules, larger or smaller segments of a cell. It goes without saying that the lysosomal enzymes with enzyomolysis action are active and act in an optimally acid pH (about 5.0), which is activated by a membrane protein pump (H+), based on using the energy emanating from ATP hydrolysis.

The dependence of the enzymes' action in these vital processes in an acid pH is vital, because this subordination protects and favors the components of cytosol, in case of lysosomal membrane injury, when the freed enzymes represent the majority and they are rapidly inactivated by the cytosalic pH which is at level 7.2. If the synthesis of the lysosomal acid hydrolases takes place in the endoplasmic reticule, then all the proteins destined for the lysosome contain mono-6-phosphate (M6P) as their marker (Covic M. et al.). This group shows, at the acrosome level (Golgi reticule trans), a specific selective receptor, through which the hydrolases are directed towards the lysosome.

The various substances resulting from the cellular and sub-cellular processes, which are useless to the body, are broken down and digested in the lysosome. Even though these processes seem to be secondary, they have their importance and they are important enough to be mentioned, as well as their means to initiate and develop in order to complete the essential reproduction processes in ferrets.

CONCLUSIONS

The beginning of the amphimixis process is the contact of the spermatozoa with the ovule and finally the penetration of a single sperm head and neck, which will cross the endoplasm, will approach and will merge with the ovule's nucleus. The external membranes' penetration process takes place with the help of acrosomal enzymes, facilitating the sperm nucleus to reach the ovule's nucleus, for the amphimixis to take place, and by mutual resorption of the two haploid nuclei, the creation of a single diploid formation, that of the future embryo. The most difficult aspect in the fecundation chain has been the capturing on film the moment of the two opposed genders' gametes amphimixis, when the two nuclei, the ovule – female and the sperm – male, become one, following their merger and mutual resorption (a process with an extremely fast evolution). The amphimixis process between the two opposed genders' gametes takes place extremely quickly, reason for which the observation of the entire process is dependent on the examination of a large number of samples, as well as luck.

Amphimixis plays a decisive role in the performance of the reproduction process, as the manner in which the process takes place can be extrapolated to other species of mammals, including human.

REFERENCES

1. *Crăciun T., Crăciun L.L.,* (1989), Dictionary of biology (in Romanian),(Ed.)Albatros,Bucharest, Romania
2. *Gluhovschi N.,* (1983), Biology and pathology of the placenta in animals (in Romanian), (Ed.) Ceres, Bucharest, Romania, 176-187
3. *Martin E.A.,* (2007), Oxford Dictionary of Medicine (in Romanian), (Ed.) ALL, Bucharest, Romania
4. *Maximilian C., Ioan D.M.,* (1984), Encyclopaedic dictionary of genetics (in Romanian), (Ed.) Științifică și enciclopedică, Bucharest, Romania
5. *Moraru L., Păunescu E.,* (1981), Encyclopaedic dictionary of immunology (in Romanian), (Ed.) Științifică și enciclopedică, Bucharest, Romania
6. *Kaaden O.*, (1984), Die Aleutenkran-heit-nenere Erkantnisse zur Atiologie und Serologischen Diagnose, Der Deut Pelt zucht, 7:109-111
7. *Oneț E.*, (1983), Viruses and animal viroses (in Romanian), Vol.II, (Ed.)Dacia, Cluj-Napoca, Romania
8. *Paraipan V.,* (1977), Diagnosis of pig fertility (in Romanian), (Ed.) Ceres, Bucharest, Romania, 108-142
9. *Paraipan V., Bucur E.,* (1981), Embryonic mortality in pigs, deficiency in vitamin E and selenium is one of the possible causes (in Romanian), Revista de zootehnie și medicină veterinară, 4:41-45
10. *Pascu, T.*, (1973), The importance of harvesting, cultivating, fertilizing oocytes "in vitro" and transplanting fertilized eggs to other organisms (in Romanian), Revista de zootehnie și medicină veterinară, 6:51-55
11. *Păstârnac N.*, (1988), Breeding ferrets (*Mustella putorius furo*) (in Romanian), Revista de zootehnie şi medicină veterinară, 2:18-27.
12. *Păstârnac N.*, (1988), Observations on the diagnosis of fertility in minks affected by Aleutian Disease in animal production (in Romanian), Revista de Zootehnie și Medicină Veterinară, 12:45-53
13. *Păstârnac N.,* (2019), The evolution of the intraovular acrosomial reaction, a vital condition in gametes' fusion and zygotes' formation, Scientifur, 43(2):63-68.
14. *Păstârnac N.,* (2019), Breeding peculiarities of farmed ferrets (Part I) (in Romanian), Veterinaria, IX(3):24-27
15. *Rusu K,* (2007), Medical Dictionary (in Romanian), 3-rd Edition, (Ed.) Medicala, Bucharest, Romania
16. *Secaşiu V., Puchianu G*. (ed), (2019), Pathology of hunted wildlife (in Romanian), (Ed.) Universitatii Transilvania Brasov, Romania, 294-300
17. *Seiciu F., Drugociu C., Boitor I.,* (1987), Reproducția normală și patologică la animalele domestic (in Romanian), (Ed.) Ceres, Bucharest, Romania, 224-228
18. *Șandor S.,* (1984), Embryology and experimental teratology of laboratory mammals (in Romanian), (Ed.) Academiei RSR, Bucharest, Romania, 64-75.