ABSTRACT

Keywords: *chicken, Lohmann, embryo, morphology, organogenesis, histology, cytochemistry, masculinization*

According to the studied bibliographical material, researches regarding the organogenesis, development, histology and cytochemistry of the genital system in chickens, has only been done on layers or mixed breeds, not on crossbreeds specialized in egg production like Lohmann Brown. From this perspective, the research protocol has been elaborated, but during the research activity, several unclear aspects have been found in the scientific literature regarding topics like the organogenesis of the female's left genital system and the regression of the right one, where the researches were undergone on an insufficient number of cases and the time periods were too distanced.

Studies regarding the embryology and morphogenesis of the female genital system on the Lohmann Brown crossbreed have never been done before. Also, a complex study, which involves researches in embryology, morphology, histology, scanning and transmission electron microscopy and the confirmation of the embryos sexes through PCR have never been conducted in chicken, the studies identified in the scientific literature being more simple and studied on fewer cases.

The research on the development of the genital system in chicks of this crossbreed is also lacking information in the scientific literature. The correlations realized between the egg production, the weight of the eggs and their components with the evolution of the oviduct are also realized for the first time, as is the development of the genital tract from the beginning of the laying period to its peak. A last point of interest, rarely found in the scientific papers is regarding masculinization in chickens, this phenomenon being studieded on three different masculinized cases, among which an ovotestis, described for the first time.

The doctoral thesis entitled *"The morphology and cytochemistry of the oviduct in chicken – Lohmann Brown crossbreed"* has been elaborated within the Doctoral School of Veterinary Medicine of the University of Agricultural Sciences and Veterinary Medicine "Ion Ionescu de la Brad" from Iassy, during the four years of study, in the period October 1st 2009 – October 1st

2013, and is structured, in conformity with the actual legal stipulation, in two main parts: the first part entitled "*Literature review*", which contains 41 pages and represents 21% and the second part entitled "*Personal researches*", that is extended on 156 pages and represents 79%.

The 1st part is divided into four chapters, and integrates the information found in the scientific literature regarding the subject of this thesis. In this part there were used 19 figures and 3 tables to illustrate the studied aspects and to detail the information.

The first chapter entitled "*Embryo development in chicken*" consists of detailed information regarding the stages of the embryo development in chicken. The second chapter entitled "*The organogenesis of the genital system in chicken*" treats the subject of organogenesis of the ovary and oviduct in chicken, reporting aspects like the progress of the left genital system and the regression of the right one. In the third chapter – "*The structure of the ovary in chicken*" information is presented, regarding the morphology of the ovary, though underlining the histological structure. Chapter four – "*The structure of the oviduct in chicken*" presents the morphology and histology of each segment of the oviduct in chicken, also referring to the crossbreed chosen for this research.

The 2nd part, "*Personal researches*" is structured in six chapters (V-X). Chapter V presents the aim and objectives of the thesis, the next chapters (VI-IX) containing the materials and research methods, the results of the research, interpretations, discussions and partial conclusions. In chapter X the final conclusions are listed, which synthetize the conducted researches. The thesis is illustrated by a number of 250 figures and 24 tables, out of which 231 figures and 21 tables are found in the second part. The study has been realized by consulting 331 reference titles.

In chapter V, entitled "*Aim and objectives*" the goal and main objectives of the thesis are listed, with the necessary activities for their accomplishment. The capital aim of the thesis is to describe the morphology of the oviduct in the Lohmann Brown crossbreed, starting with the sexual differentiation, in the 7th day of embryonic development, towards the peak of the laying period in the adult chicken.

Another objective is the research of the morphological and cytochemical aspects of the development of the genital system in this crossbreed specialized in egg production, to underline the changes that appear as a consequence of genetic selection. A different objective is the SEM and TEM research, that allow us to have a better view on the structural details of the samples taken into study. To highlight the performances of the studied crossbreed, we evaluated its productive performances in the intensive growth system. A last objective is the research of the masculinization phenomenon in the chicken, which is rarely found in the scientific literature.

The research in chapter VI, titled "*The organogenesis of the genital system in Lohmann*

Brown chicken embryos" has been realized by harvesting samples from a total of 247 Lohmann Brown chicken embryos, 127 incubated in Romania and 120 obtained from the Virology department of the Faculty of Veterinary Medicine, of UGent, Belgium. Relevant macroscopic pictures have been taken, and measurements of the ovaries and the Müllerian ducts, to highlight their development and regression; and also the eggs and embryos were weighted to underline the embryonic evolution. Samples were harvested for the histological exam, SEM, TEM and PCR.

The biological samples were harvested differently, depending on the age of the embryo and stained HEMB, PAS, Novelli and Gömöri. The Gömöri stain highlights the reticulin fibers, this being a way to differentiate very easily the testicle from the ovary.

Starting with the 7th day of embryonic evolution, the development of the gonads into testicles, in homozygous embryos, ZZ and into ovaries in heterozygous embryos, ZW starts. At this embryonic age, the left gonad is larger than the right one, regardless whether it is a testis (in male embryos) or ovary (in female embryos). The structure of the female gonads is similar, observing an epithelial germinal layer with somatic cells and numerous germinal cells and a medullar area formed of medullar cords amongst which many mesenchymal cells can be found.

During this period, both Wolff ducts and Müllerian ducts are distinguishable, and have a common, parallel, tract. With the SEM, the Wolff and Müllerian ducts from the left side can be noticed, surrounded and separated by a thick layer of mesenchymal cells. The Wolff duct appears elongated, with an open lumen and lower cells, unlike the Müllerian duct which is closed and has a round shape with taller epithelial cells.

The ovaries of the 8 days embryos can be differentiated histologically, the right from the left one, and from the testicles. Comparing the structure of the two ovaries, left and right, it is noticed that, the left one develops a well defined cortical area, unlike the right one, whose cortex doesn't proliferate.

In the 9th day, the left ovary continues to develop based on the proliferation of the germinal epithelium in the cortical area, the two regions becoming more and more different from one another. In the testicles, the sexual ducts are disseminated across the organ, giving it a labyrinth aspect, without differentiating the two regions (cortex and medulla). The left Müllerian duct is well highlighted close to the metanephros, still being surrounded by a thick layer of mesenchymal cells. The Müllerian epithelium is simple, formed of cubic cells, which present signs of mitotic division.

In the 10th day, the right Müllerian duct starts to regress and to have a different structure, compared to the left one. The left Müllerian duct epithelial cells appear tall, leaving a tight lumen, compared to the ones of the right-sided duct which are small and flattened, like the mesenchymal cells.

In the 11th day, the left Müllerian duct can be seen with SEM, being formed of a simple epithelium, with low cells, having a height of 15-17 µm, and presenting microvilli-like cytoplasmatic extensions.

In the 12th day, the left Müllerian duct continues to develop, the epithelial cells being taller, structuring a simple prismatic epithelium. The right one presents epithelial cells of small dimensions, the duct losing its identity within the mesenchymal cells layer.

In 13 days old embryos, the right ovary appears structured just by the medulla, with many vacoules and fragmentations of the sexual cords. The size of the entire right gonad is smaller than the medulla of the left ovary. The differences between the testicles and the two female gonads consist in the development of the sexual cords in the entire mass of the testis, organized in a specific, labyrinth-like form. The seminal tubes will be formed from the sexual cords.

In 14 days old embryos, the female genital system continues to develop on the left side, the ovary having a medium length of 4,76 mm and the width of 1,66 mm. The right gonad decreases, getting to the length of 2,73 mm and a medium width of 0,53 mm. The left Müller duct reaches the medium length of 16,86 mm and the right one 6,3 mm.

At 15 days, the reminiscence of the right Müller duct can be seen, which keeps its shape and dimensions, without indentifying any more epithelial cells or an epithelium, just an amorphous cellular mass.

At 16 days, histologically, the distancing of the left ovary from the kidney can be noticed and also the elongation of the medulla. The ovary remains attached with the hilum to the kidney, where large blood vessels can be found. In the cortex there are numerous oogonia which are organized in primordial follicles.

At 17 days, the left ovary changes its shape and position related to the kidney. Also the intense elongation of the medullar cords can be noticed, these forming large spaces in the medullar region. The regression of the right ovary is noticeable as well, which presents only the medullar area, elongated as the one of left ovary, and at a certain distance from the right kidney.

In the 18th day, the left Müller duct grows in diameter, the epithelium being composed of more and more prismatic cells that structure a large lumen. Around the epithelium there is a thinner and thinner layer of mesenchymal cells.

In the 19th day, using SEM, the cortical structure of the ovary can be observed, being formed of numerous oogonia and primordial follicles.

In the 20th day, the left ovary has the medium length of 7,36 mm and a diameter of 2,56 mm, the right one having the length of 2,23 mm and a diameter of 0,43 mm (6 times smaller than the left ovary). The medium length of the left Müllerian duct is 28,5 mm, compared to 3,73 mm, the length of the right duct. These values are confirmed by the histological images, which present

a well developed left ovary, opposed to the right one. The cortex of the left ovary is thinner in the middle areas, with a higher proliferation at the ends. The oogonia continue the process of gathering follicular cells and structure primary follicles. The right Müller duct regresses caudally towards the cloacae, being noticed like a mass of mesenchymal cells the accompany the right Wolffian duct. The left Müllerian duct presents the first small primary folding of the epithelium.

The left ovary grows from 3,3 mm length at the age of 7 days to 7,36 mm length and 2,56 mm width, at 20 days of age. The right ovary grows from 3,2 mm length at 7 days to 3,43 at 8 days, after which it regresses to 2,23 mm length and 0,43 width at 20 days. The left Müller duct grows in length from 6,3 mm at 7 days to 28,5 mm at 20 days of age, unlike the right one which from 6,3 mm at 7 days, measures only 3,73 mm at hatching.

The relation between the embryos' weight and the eggs' weight, depending on the sex, shows that the female embryos develop faster than the males.

The PCR exam confirmed the sex identification, based on the gonads' morphology.

Using TEM, on the right Müller duct's cells, a cytoplasmic vacuolization can be noticed, with the presence of pyknotic nuclei, condensed chromatin and the retraction of the nuclear membrane, signs of necrobiosis that confirm the regression of this duct. Regarding the ultrastructure of the left Müller duct, we can notice the cilia genesis in the ciliated cells; the secretory cells with a clear cytoplasm, having rare cytoplasmic organelles, meaning the cytoplasmic components are in the organizing faze of the necessary for an intense metabolism.

The research in chapter VII, entitled "*The development of the genital system in Lohmann Brown chicks*" was conducted on individuals raised in the biobase of the U.A.S.V.M, Iassy, from one day of age to 18 weeks - the age when laying starts. The samples have been harvested from 51 birds, examined histologically and observed using the optic microscope Motic B1-211A with the Moticam 1000 video camera.

During the first 10 weeks from hatching, the development of the female genital system in the crossbreed considered for study is very slow. The ovary presents the two regions clearly delimitated: cortical and medullar. In the cortical area there are numerous follicles in the process of proliferation and maturation and stromal cells. Primary follicles can be observed, which accumulate nutritive vitellus and form secondary follicles starting with the 5^{th} day. In the oviduct, the mucosa presents folding, up to 10 weeks only primary folds can be distinguished with simple prismatic epithelium and mesenchymal cells at the base.

Starting with the 11^{th} week, histological differences can be seen between the 5 segments of the oviduct regarding the evolution of the mucosa folds and the musculosa layers. The secondary folds of the mucosa can be seen starting with the 12^{th} week in the magnum and the 14^{th} week in the other segments of the tube. The musculosa appears to be developed specifically

for each segment, presenting the two smooth muscle cell layers, starting with the 15th week from hatching.

In 15 weeks old chicks, rare tubular glands appear in the lamina propria of the magnum, isthmus and uterus, leaving large spaces of connective tissue. They develop and present secretion in the cells in the 17th week and in the oviduct's lumen in the 18th week - the start of the laying period, demonstrating the precocity of this crossbreed.

At 16 weeks of age, the first tertiary follicle appears in the ovary, until the age of 18 weeks, the left gonad having the characteristic aspect of a grape bunch, specific for adults.

At 18 weeks of age the segments of the oviduct have all the structures well developed and functional, being formed of a mucosa, submucosa, musculosa and serous membrane.

The research in chapter VIII, entitled "*The morphology and cytochemistry of the genital system in chicken – Lohmann Brown crossbreed*" has been realized using a number of 25 Lohmann Brown chickens, from which samples have been harvested weekly, between the age of 19 and 29 weeks and depending on the position of the egg in the oviduct for the ones that are at the peak of egg production. For this research, the ovarian follicles larger than 10 mm were measured and counted, in order to observe the evolution of the ovarian status; the segments of the oviduct were also measured to highlight their development and to correlate this with the growth of the eggs, their components and the laying percentage. Samples from each segment were harvested for histological exam, cytochemical exam, SEM and TEM. We also performed measurements on the main histological structures of interest from every segment (height, diameter and number of the main folds, epithelium, secretion granules, cilia, nuclei, nucleoli, tubular glands, glands lumen, glandular cells, musculosa), to be able to observe their evolution but also the differences in structure between the segments. Histological stains were performed for orientation: HE and HEMB and cytochemical staining: PAS, PAS Alcian blue pH 2,5 (for glycoproteins), Masson, van Gieson (for collagen) and von Kossa (for calcium deposits).

The ovary appears very well developed, with 6-9 yellow follicles on its surface measuring over 10 mm and many small, white follicles.

On the surface, the ovary is covered with a simple, germinal epithelium, formed of cubic cells which flatten because of the pressure exerted by the evolution of the follicles. In this case, the epithelium can become squamous with flattened nuclei. In this epithelium, the cubic cells have different heights, so that, at the surface, the epithelium has a tooth-like appearance. Under the germinal epithelium there is a layer of connective tissue which forms the tunica albuginea. In the cortex of the ovary, next to the tunica albuginea, numerous follicles of different dimensions can be seen, developing and accumulating vitelline granules, but also postovulation follicles or atretic follicles. Histologically, the tertiary follicle is structured of: oocyte, corona radiata,

perivitelline membrane, granulosa, basal lamina, internal theca and external theca.

After ovulation, the follicular walls flatten and get thicker because of the cell concentration in the granulosa and the internal theca, forming a structure similar to the corpus luteum in mammals. Using SEM, the cubic epithelium and follicles in different stages are seen on the surface. On the inside, small oocytes and follicular cells are observed.

The oviduct presents the 5 regions: infundibulum, magnum, isthmus, uterus and vagina which develop intensively from the beginning of the laying period (19 weeks) to its peak (29 weeks).

The mucosa of the infundibulum funnel presents in the epithelium many ciliated cells and rare secretory cells, the lamina propria lacking tubular cells. The musculosa is well represented but the orientation of the fibers is different from the other segments, noticing groups of smooth muscular fibers with various orientations.

With SEM numerous ciliated cells are observed and very rare secretory cells in the epithelium, without secretion granules in the lumen.

The neck of the infundibulum presents primary folds, with secondary and tertiary folding. The tubular glands are rare in the lamina propria of the mucosa, but there are many secretory PAS positive cells in the surface epithelium. This aspect is highlighted also with SEM, observing rare ciliated cells and many secretory ones, between which there are openings of the tubular glands from the lamina propria. Also, a secretion organized in granules and filaments can be seen in the lumen.The main fold in this segment has approximately 1185 µm in height and 60 µm in diameter. The structure of the cilia and the intercellular junctions realized by desmosomes can be observed. Secretory cells containing electronodense secretion granules that confirm their protein nature can also be seen.

The magnum presents very large and thick primary folds, due to the tubular glands which are full of PAS positive secretion for the secretion of the albumen. These glands elaborate the largest part of the egg, the albumen. Out of the total mass of an egg - 50 g, the albumen represents approximately 62% of the weight, meaning 31 g. So, the mucosa has the role of secreting 31 g of albumen in approximately 3 hours, while the oocyte passes through this segment. This is why the magnum is the longest part, having 47 cm in the crossbreed considered for this study and the mucosa presents the most developed folds. On this segment, the primary fold of the mucosa has a medium height of 3156 µm and a diameter of 1145 µm. The two types of cells from the epithelium, ciliated and secretory, alternate; none of them predominating, the report being approximately equal. On the base of the folds, there are more secretory cells, while towards the top of the fold, the ciliated ones are mostly seen.

With SEM the aspect of ciliated and secretory cells can be observed, and also numerous

openings of the tubular glands in the lumen and a lot of secretion in the lumen.

With TEM we observe the ciliated cells with many cilia at the apical pole, both in longitudinal and transversal secretions. The nucleus of these cells is elongated, with nucleolus, situated in the superior third part of the cell. In the plasma of the nucleus the granular chromatin and the heterochromatin can be observed. The secretory cells, viewed with TEM, have a well-developed endoplasmic reticulum situated around the nucleus. The nucleus membrane is fringed to increase the exchange surface between itself and the cytoplasm. On the apical pole of the cell there are condensed vesicles of the Golgi apparatus with a dense content to the flow of the electrons, which proves an intense secretory activity.

In the isthmus, folds similar to the magnum's mucosa can be seen, but they are less tall and thinner. In this segment the shell membranes are formed, the internal and external ones, for approximately one hour. The secreted material has an approximate weight of 0,6 g for the 50 g eggs, representing 0,12% of the egg's total weight. A particular aspect of the isthmus rigards the secretion at this level, which is organized specifically, as PAS positive filaments or granule rows.

At this level, the mucosa has a medium height of 2373 µm and a diameter of 733 µm.

With SEM, the almost equal proportion between the two types of cells from the surface epithelium is observed and also the form of the secretion driven to the apical pole of the cilia, into the lumen.

Ultra-structurally, the secretory cells present a serrated nucleus, with two nucleoli, as a sign of an intense activity in the ribosome synthesis. Near the nucleus there are elements of the granular endoplasmic reticulum and its cisterns filled with secretion which will generate transfer vesicles.

The uterus presents a very developed musculosa, formed of the two layers: inner circular and outer longitudinal, the internal one being much more developed, measuring 548 µm. The mucosa's folds present a different aspect at this level, being more flattened, leaf-shaped, with a height of 2350 µm and a diameter of 307 µm. The secretory cells, cup-shaped, of the epithelium have the role of secreting materials which complete the structure of the eggshell, the mineralization process being highlighted with von Koss stain.

With SEM it is observed that the elaboration of the secretion that forms the eggshell is realized as granules and also filaments, transforming into an amorphous mass which totally covers the surface epithelium. Ultra-structurally, the secretory cells present a nucleus and are rich in metabolically active chromatin which is accessible to RNA polymerase II. The heterochromatin is shaped in small blocks and the facultative X chromatin is present on the internal side of the nuclear membrane, which confirms the female sex.

The vagina has a length of 14 cm and a diameter of 8,5 cm. The mucosa presents a particular aspect, with tall main folds, many small secondary folding that give a tooth-like aspect. At this level, as seen also in the infundibular funnel, in the lamina propria there are no tubular glands. This aspect makes the folds very thin, the structure of the lamina propria and submucosa being made of just connective tissue and blood vessels. In the epithelium predominate the ciliated cells, in the detriment of the secretory ones, the ratio being 2-3 ciliated cells to one secretory cell. The secretory cells are seen more frequently at the base of the main folds, presenting in the cytoplasm PAS positive secretion granules. The vagina's musculosa has the internal circular layer well developed. The muscular fibers form groups surrounded by conjunctive tissue and blood vessels. With SEM, both ciliated and secretory cells can be seen, and also a secretion organized as granules and filaments in the lumen of this segment.

Ultra-structurally, the process of exocytosis through which the secretion granules of the secretory cells from the epithelium are discharged to form the cuticle, is highlighted. The secretory vesicles have different sizes, with a dense content for the electrons flow, which shows their protein-based nature.

The data regarding the egg production of this crossbreed show that the age at the start of the laying is 18 weeks, which highlights the precocity of this crossbreed opposed to the layer breeds Leghorn (19-21 weeks) or mixed, Rhode Island (25 weeks), or Leghorn (19-21 weeks).

This crossbreed reaches the laying percentage of 97%, higher than any other crossbreeds or layers, confirming the quality of these chickens. We highlighted a correlation between the development of the oviduct's segments and the growth of the laying percentage, of the egg's weight and it's components.

After this research, the hypothesis according to which, consecutive to the genetic selection to obtain a higher quantity and quality egg productions the morphology of the oviduct modifies, is confirmed.

In chapter IX, entitled "*Researches regarding masculinization in chickens*" the research material consisted in three layers which manifest an unspecific behavior for females or specific for males. In the first case, the samples consist in the anamnesis information regarding the chicken's behavior and macroscopic images realized after the necropsy. The samples for the second case consist also in anamnesis data, images and video recordings, histological samples from the ovary and oviduct, but also hormonal analysis for the quantification of cortisol, progesterone, testosterone and oestradiol levels in the blood, for the researched chicken and also a group of five other chickens and a rooster that form a control group.

For the third case, blood samples have been taken for hormonal determinations and also tissue samples for the histological exam.

All three cases taken into study present behavior changes, the exaggerated development of the combs, bibs and spurs, much more colored feathering, all these representing secondary sexual characters in males. The female genital tract presents morphologically as a tumor in the first case; an ovarian congestion and a cyst-like structure developed from the reminiscences of the right Müllerian duct in the second case (the left oviduct did not show morphological differences), and for the third case, an ovotestis and the left oviduct with a normal morphology.

In the first case, the tumor is thought to generate endocrine disruptions which cause hormonal changes that led to the masculinization. In the third case, the testicular part of the ovotestis is responsabile but, in the second case, no hints were found in respect of the reason of the high testosterone level.

Histologically, in the second case, the congestion of the ovary has been confirmed and there are many primary and secondary follicles in the course of atresia, with a folded wall. In the oviduct of the same case, an involution of the lamina propria of the magnum was noticed, and also a histologically modified appearance, presenting primary folds that are thinner and many secondary and tertiary folding. In the isthmus, we found cystic tubular glands, the aspect of the other oviductal segments remaining unmodified.

The histology of the ovotestis, for the third case, shows a region similar to the cortical area of the immature ovary at the exterior, representing the ovarian part and the testicular part similar to the medullar area, on the inside. The ovary presents a tertiary follicle and many primary and secondary ones in necrobiosis. The testicular structure presents seminiferous tubules with Sertoli cells and spermatic immature cells that present pycnotic nucleus - sign that they will not develop further into spermatozoa. In the angular spaces of the seminiferous tubules, Leydig cells are noticed, with the role in secreting testosterone, which explains the masculinization for this case. The oviduct of this chicken presents a structure morphologically close to the chicken of same age group.

The level of testosterone in the birds taken into study is 5 to 6 times higher than the average of the chicken in the control group and 3 to 4 times lower than in the case of the rooster, which explains the expression of masculine secondary sexual characters.

The oestradiol level for the second case is under the values of the other chickens, which shows an inhibition of the ovarian activity that could have led to the evolution of a testosterone secreting structure.

The masculinization in chicken appears as a consequence to the lowering or stopping of the left ovary activity, regardless the cause.

Chapter X contains the final conclusions that systematize the results of the research.