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Original Article

Daily Egg-Cycle in Japanese Quail: Serum Biochemistry, Bones, and Oviduct Changes

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ABSTRACT

This study described changes in the serum biochemistry, morphology of genital organs, long bone, and eggshell during the daily egg formation cycle in Japanese quails. Sixty quails (18-wk) were distributed in 6 groups according to hours post-oviposition (POV): 0 hr POV (16h00), 2 hrs POV (egg in magnum), and 4, 8, 14, and 20 hrs POV (egg in uterus). The magnum had higher relative weight before the next ovulation (20 and 0 hr POV), and its tubular glands showed functional variation through periods: abundant eosinophilic, PAS+, and negative Alcian blue secretion at 0 and 2 hrs, empty glands aspect at 4 hrs, and filled again at 20 hrs POV. Serum albumin and total Ca had the highest value in the 2 hrs group, and the lowest in 8 and 14 hrs groups. Egg-cycle period affected the Ca% of the medullar bone of the femur and tibiotarsus, with the lowest mean at 14 hrs POV (06h00), and the highest mean after oviposition (0 hr POV), showing the recovery of Ca stores in long bones for the next egg cycle. Analysis of the eggshell using scanning electron microscopy evidenced that palisade layer formation starts during the night (8–14 hrs POV), and most parts are secreted during the day period. In conclusion, eggshell secretion in light periods, high magnum activity and medullary bone Ca deposition during midday and afternoon, as well as the ovulation/oviposition in the afternoon, are the main characteristics of the distinct physiological aspects of the egg cycle in quails.

INTRODUCTION

Quails are taken to be comparable to laying hens in reproductive physiology, but their egg-laying period places them at a different physiological moment. The general metabolism of egg formation in quails is similar to laying hens, but with ovulation and oviposition in the afternoon, and most calcification of the shell period during the day (Holm *et al.*, 2001; Bar, 2009). The morphological description of genital organs in quail is also general and does not explore the differences during functional phases.

Daily egg-cycle in avian females in the active reproduction period lasts around 24–25 hours, including ovulation of the oocyte in the ovary and production and secretion of the egg part in the oviduct (Nys *et al.*, 2021). The calcified eggshell with a great amount of calcium is secreted by uterus mucosa during the hours of eggshell formation (Whitehead, 2004). In this sense, female birds develop an integrated metabolism among bones, intestine, kidneys, and hormones to maintain blood calcium levels during the daily egg formation cycle (Van De Velde *et al.*, 1984; Kim *et al.*, 2012).

Avian eggshell formation is one of the most rapid biomineralization processes know in nature. This process occurs in the lumen of the uterus, is based in a large amount of daily calcium and bicarbonate secretion

(Nys *et al.*, 2021), and can be divided into 3 distinct phases. The first 10 hours after ovulation make up the initial phase, with the slow secretion of $CaCO_3$ to the uterine lumen. The next 12 hours make up the rapid-growth phase with linear deposition of eggshell; and the terminal phase, just 2 hours before oviposition, is characterized by the arrest of calcification and the deposition of the cuticle. The rapid-growth phase demands a great amount of Ca from blood and occurs during the night in laying hens. Calcium is provided directly by ionic blood Ca, and the amount necessary to an eggshell in hens (2 g/egg) is provided directly by the diet, with one-third (30-40%) being mobilized from the bones (Nys *et al.*, 2021), especially from medullary ones (Bar, 2008). In quails, with oviposition in the afternoon, the largest period of shell formation (rapid-growth phase 10-22 hrs post-ovulation) starts during the night, but most of it happens in light/day hours, when females have access to food and calcium sources from the diet.

Long bones of laying bird females have a special source of calcium, the medullar bone, from which Ca is removed for transfer to the blood and then to the uterus (Bar, 2009). In this process, estrogen plays a critical role in Ca metabolism, with activation of the osteoclasts, liberation of the parathyroid hormone (PTH), and intestinal absorption. These effects result in an increment of blood calcium availability for shell formation (Bar, 2009; Kim *et al.*, 2012).

The continuous process of laying over time structurally affects the bones. In laying hens, skeletal disorders are a welfare and economic concern for the poultry industry. Osteoporosis is the main problem caused by the progressive use of structural and medullary bones for eggshell formation during the bird's life (Whitehead, 2004). However, this is not very well studied in quails and no research has been found on osteoporosis or leg problems for this species.

Although extensive research has been conducted in avian reproduction and physiology, few efforts have been made to describe whether quail genital organs and medullary bones follow the same mechanisms of birds with a morning oviposition metabolism. This characteristic suggests that in this species the mechanisms of calcium mobilization of the intestine and bones, as well as the process of production and synthesis of parts of the egg, follow different pathways. Considering these aspects, this study described morphophysiological changes in parts of the oviduct and calcium metabolism in blood and long bones in Japanese quails throughout the egg formation cycle, from the time of ovulation through the phases of shell formation and eggshell deposition in the uterus.

MATERIAL AND METHODS

Birds and Ethical Approval

The experiment was approved by the Committee of Ethics on the Use of Animals (CEUA) of the State University of Maringa, approval number 7006280815.

Sixty18-wk-old female Japanese quails (*Coturnix coturnix japonica*) were used. Hens were standardized by weight (170 ± 8.5g) and egg production (95%) and housed in individually galvanized laying cages (17.5 × 14.5 × 9.0 cm). The light program was 17 hs (natural + artificial, light 05h30 to 22h30) during summertime, with a daily average temperature and humidity of 22.96°C and 89.63%, respectively. The diet followed the requirements for quails in the laying phase (Rostagno *et al.,* 2011), based on corn and soybean meal (CP 18.80%, ME 2,800, calcium 2.92% and phosphorus 0.30%), with food and water *ad libitum*.

The experimental design was composed of 6 groups (treatments) according to the physiology periods of the egg formation cycle (0, 2, 4, 8, 14, and 20 hrs post-oviposition (POV)), with 10 replicates (quail was considered as an experimental unit). Each quail was observed daily to determine the exact oviposition time and quails were distributed in treatments according to the time of egg laying, (Table 1, Figure 1). All females laid eggs around 16h00.

Table 1 – Distribution of quails in the groups based on the period post-oviposition (POV), description of egg in the oviduct, and estimated day-time of each group (dark period 22h30 to 05h30).

Estimated day time[1]	Group	N	Description
16h00	0 hr POV	10	Post-oviposition, just before the next ovulation
18h00	2 hrs POV	10	Egg in magnum and albumen secretion
20h00	4 hrs POV	10	Egg in the uterus (inactive phase)
24h00	8 hrs POV	10	Egg in the uterus (beginning of active phase)
06h00 (next day)	14 hrs POV	10	Egg in the uterus (active phase)
12h00 (next day)	20 hrs POV	10	Egg in the uterus (final egg formation)

[1] Day time when quails were euthanized after the last observed oviposition.

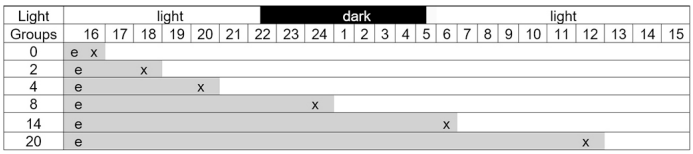

Figure 1 – Scheme of group distribution after the oviposition of an egg (e) around 16h00. "X" represents the estimated day period of post-oviposition (POV) when females were analyzed in each group.

Tissue and Blood Collection

All quails of each group (n = 60) were weighed (live body weight, g) and blood collected by venipuncture of the jugular vein in a tube without anticoagulant, centrifuged at 3,000 rpm for 15 min to obtain 2 mL of blood serum. Serum samples were frozen at –20°C and analyzed by Gold Analisa® commercial kits for the determination of total calcium (Ca), phosphorus (P), total protein, albumin, and alkaline phosphatase levels in a UV-VIS Evolution 300-spectrophotometer. Ionic calcium was determined by the formula:

$$CaI = 6 \times Ca - (0.19 \times Prot) + A/3)/(0.19 \times Prot) + A + 6$$

where the units are CaI in mg/dL, Ca (calcium mg/dL), *Prot* (total protein g/dL) and A (albumin (g/dL) (Gold Analisa® instruction kit).

Quails were subsequently anesthetized with an intraperitoneal barbiturate injection (sodium thiopental 10mg/kg body weight) and killed by cervical dislocation, and the bones and organs were collected and weighted (liver, ovary, and oviduct). The vitellogenic ovarian follicles (yellow) were denominated as a function of their size, from largest to smallest, as F1, F2, F3, F4, and the follicular diameter were determined.

The segments of oviduct magnum, isthmus, and uterus were isolated and weighted. The relative weight (%) of organs and oviduct segments were calculated based on body weight. The total number of magnum folds were determined macroscopically.

Histological analysis

Samples from the middle portion of the magnum and uterus were collected (0.5 cm), attached to a Styrofoam surface with pins to expose the mucosa, and immersed in a 10% formaldehyde fixative solution, with a 0.1M phosphate buffer pH 7.4. The samples were histologically processed in a routine, paraffin-embedded cut (3 µm) and stained with hematoxylin and eosin (HE) for histological analysis. Cuts from the same samples were also analyzed using histochemical reactions of periodic acid-Schiff (PAS) to identify the secretion of neutral glycoproteins, and Alcian blue pH 2.5 (AB) to identify polysaccharides.

To determine the height, width, and area of the folds of the magnum and uterus, digital images were obtained using light microscopy (Motic BA400) coupled with the Moticam 2500 5.0MP USB 2.0 image capture camera, and images were analyzed in the Motic software Images plus 2.0 (Motic China Group Co. Ltd. software). The height and width of the magnum and uterus folds were measured for at least 05 folds/quail/segment/treatment (magnification 4x), while the percentage of the ciliated and secretory cells of magnum were obtained in 300 cells/quail/treatment (magnification 100 x), and the epithelial height of mucosa cells in the magnum and uterus were obtained in 50 cells/quail/treatment (magnification 100 x).

Scanning Electron Microscopy (SEM) of eggshell

In females of groups 4, 8, 14, and 20 hrs POV with eggs in development inside the oviduct segments, eggs were weighed and their eggshell with shell membrane were prepared for analysis by scanning electron microscopy. Eggs were fixed with glutaraldehyde 2.5% in a phosphate buffer 0.1M pH 7.4. After fixation, samples from the eggshells were obtained from strips or fragments of the equatorial region of the eggs using a razor blade. Samples were then washed, and dried in an oven at 37°C for 72 hrs. Eggshell samples were stuck in an aluminum stub using a specific double-sided tape product with a vertical orientation or a cross-sectional view. The stubs with samples were prepared for scanning in the sputter coater with a gold target, and metallized for 60 seconds (s). Standard procedures were used to set up the Shimadzu scanning electron microscope (model Superscan SS-550, Hadono, Kanagawa, Japan), the correct filament saturation and proper stigmatization were performed, as well the control of contrast and brightness of images. Images were obtained and saved as grayscale TIFF files (Fathi *et al.,* 2006).

Bones analyzes

Seedor Index

Femur and tibiotarsus were dissected, weighed, and measured with a pachymeter at their greatest length. Data were used to determine the Seedor index dividing the result of the weight of the bone by its length. Seedor Index = Weight (mg) / Length (mm).

Bones Mineral Density (BMD)

The same bones were x-rayed along with the aluminum penetrometer (with 8 levels from 1 to 8 mm) at the CDVET - Veterinary Diagnostic Center, in a radiographic equipment Tecno-Design 300/150 with the technique Kv 40, MA 50, and MAS 40 in a digital radiographic film CR - Carestream, Direct View Vita CR veterinary. Radiographswere taken to determine bone density in the region of the diaphysis of each bone using the tool "Histogram" of the Adobe Photoshop CC software, since it was the same area that received the application of the necessary force to the bone in the resistance test, using the penetrometer levels as a reference (Berti *et al.*, 2006).

Bone Strength

The right bones were used for measuring the maximum strength of bone breakage. Bones were analyzed at the Food Engineering Laboratory in Brookfield Engineering Laboratories Inc. MA, USA, with the TexturePro CT V1.4 build 17 software. The parameters used were a compression test with 5% deformation, Trigger 500g, test speed 0.05 mm/s, TA7, and T7TPB device. The device was the three-point bend rig, where the bones were positioned with support only in the region of the bone epiphyses, and the probe force was applied in the diaphysis region, and the force applied at the time of bone rupture (kg) was measured (Bonagurio *et al.*, 2020). The variables hardness (resistance to permanent deformations) and fractureness or fractureability (fragility, susceptibility to fracture, and deformability before breaking) were also obtained.

Mineral Contents

The left bones were used for mineral contents analyses. Bones were cut longitudinally to extract the medullary bone (with the marrow bone). Medullary bone was analyzed separately from the rest of the bone. The ashes, calcium (Ca), and phosphorus (P) percentage were obtained according to Silva & Queiroz (2006). Samples were calcined in a muffle at 600°C for six hours and after the cooling sent for weighing. To decomplex the hydroxyapatite crystals and release minerals, 10 ml of hydrochloric acid (6M) was added to the ash and placed on a heating plate. The solution was subsequently evaporated in the exhaust hood until completely dry. The precipitate was then dissolved by adding distilled and deionized water until the volume of the filtered solution reached 100ml.

The solution was used to determine mineral contents. Calcium concentrations were analyzed using a Varian Atomic Absorption AA240FS spectrophotometer with the SpectrAA software from Victoria, Australia; and phosphorus concentrations were determined by colorimetry on a UV-VIS Evolution 300-spectrophotometer.

Statistical analysis

Based on the results, normally distributed variables were subjected to ANOVA using the Tukey test, and not normally distributed parameters were analyzed by the Kruskal-Wallis test, using the R Studio software with a 5% significance level.

RESULTS

There was a significant effect of period of POV on body weight (p=0.011), weight of egg in oviduct (p<0.001), and relative weight of ovary + oviduct (p<0.001), magnum (p<0.001), and isthmus (p<0.001) (Table 2). Quail body weight was higher for the 20 hrs POV group, and lower in the 0 hr POV one. Relative weight of ovary + oviduct (without egg) was higher in the14 and 20 hrs groups.

Data from the 4 largest vitellogenic ovarian follicle diameters are shown in Table 2. The follicles in general had different sizes during the 24-hour cycle as a function of follicular hierarchy (Figure 2), with the largest diameter in the periods closer to the oviposition (0 hr group), and the smallest in the period 2 hrs POV (Table 2).

Magnum relative weight (%) had the highest values in females at 20 hrs POV, and immediately before the next ovulation (group 0 hr), while in isthmus,

Table 2 – Means of the body weight and eggs inside oviduct segments weight (g), relative weights (%) of the ovary + oviduct, magnum, isthmus, uterus liver, and follicular diameter F1 to F4 (mm) in Japanese quails during the daily egg-laying cycle.

Variables	Time post-oviposition						Means	SEM	*p value*
	0 hr	2 hrs	4 hrs	8 hrs	14 hrs	20 hrs			
BW (g)	161.40^{b}	168.30^{ab}	171.10^{ab}	165.40^{ab}	163.10^{b}	177.70^{a}	167.96	1.35	0.011
Egg in oviduct (g)	-	-	06.63^{d}	09.50^{c}	10.39^{b}	11.50^{a}	09.66	0.11	<0.001
Relative weight (%)									
Ovary + Oviduct	08.16^{d}	09.35^{c}	09.81^{c}	11.89^{b}	13.54^{a}	14.44^{a}	11.25	0.11	<0.001
Magnum	02.33^{a}	01.76^{b}	01.60^{b}	01.61^{b}	1.84^{b}	02.21^{a}	01.90	0.03	<0.001
Isthmus	00.53^{a}	00.50^{abc}	00.47^{abc}	00.46^{bc}	0.43^{c}	00.55^{a}	00.49	0.01	<0.001
Uterus	01.27	01.32	01.27	01.30	1.39	01.39	01.32	0.03	0.530
Liver	03.71	03.36	03.55	03.54	3.46	03.52	03.53	0.05	0.943
Follicular Diameter (mm)									
F1	18.31^{a}	15.99^{c}	16.39^{bc}	16.92^{abc}	17.67^{ab}	18.36^{a}	17.25	0.15	0.012
F2	14.50^{ab}	11.55^{c}	12.8^{bc}	12.39^{c}	14.26^{ab}	15.38^{a}	13.40	0.18	<0.001
F3	10.86^{a}	07.55^{b}	08.17^{b}	08.09^{b}	10.10^{a}	10.96^{a}	09.26	0.17	<0.001
F4	07.26^{a}	04.69^{c}	05.02^{c}	05.54^{bc}	05.89^{abc}	06.77^{ab}	06.03	0.16	<0.001

^{abc} Means with different letters within the same column differ significantly in the Tukey test (p<0.05). SEM: Standard error of the mean

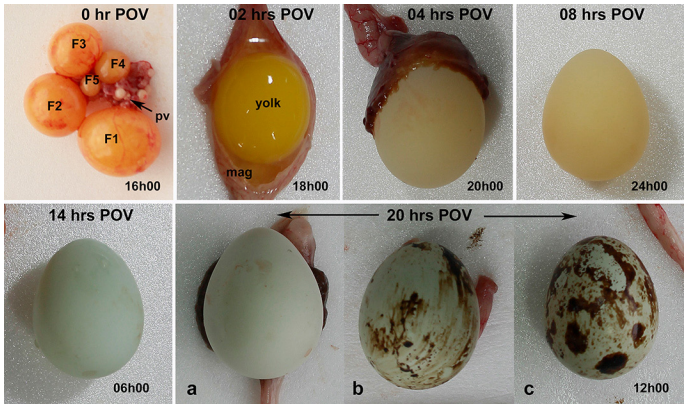

Figure 2 – The daily egg-laying formation cycle. At the moment of oviposition (0 hr), follicular hierarchy (F1-F5), preovulatory follicle (F1), and previtellogenic follicles (pv) are visible. At 2 hrs post-oviposition, the magnum (mag) is secreting albumen (al) around the yolk. Between 4 and 20 hrs POV, the formation of membrane fibers and eggshell occurs. At 20 hrs, we can observe eggs in the final period of eggshell formation in some quails, with carbonate calcium deposition (a), pigment deposition (b), and the final egg with cuticle (c).

it occurred after 14 hrs POV (inactive phase). The magnum data showed that there was no difference in length between the periods studied, with a mean of 14.49 cm and 11 longitudinal macroscopical folds.

Macroscopically, the uterus was characterized by an expanded oviduct region located caudally to the isthmus, with dark brown mucosa and indistinguishable folds, without significant differences (p>0.05) in uterus weight during daily egg-cycle.

The eggs of all females in the 2 hrs POV groups were in the magnum, and in females from 4 to 20 hrs POV eggs were already in the uterus. Egg weight increased during the period inside the uterus from 6.63 to 11.50 g (Table 2).

Histological aspects

Histologically, magnum folds had rounded edges, with 1.19 mm in height and 0.68 mm in width, revested by epithelium with the highest height at 2 hrs, and the lowest after 14 hrs POV (Table 3). Magnum epithelium was composed by ciliated cells with a central nucleus, and by secretory cells with a basal nucleus and cytoplasmic granules PAS+ and AB+, without HE staining (Figures 3, 4). The same proportion of secretory and ciliated cells (1:1) was observed during egg-day cycle (Table 3, Figures 3B, 4H).

The folds of the magnum were filled with tubular glands characterized by cytoplasmatic granules and luminal eosinophilic content, PAS+, and AB- (Figures 3, 4). In the period 2 hrs POV, the egg was present in the magnum and these glands were fuller. The content was copiously secreted by the glandular lumen and from these to the lumen of the magnum through ducts to form albumen. The albumen resulting from the secretion of superficial secretory cells and glands in the magnum lumen was eosinophilic (Figure 4J),

Table 3 – Morphometric values of the magnum and uterus in Japanese quail during the daily egg-laying cycle.

Variable	Time post-oviposition						Means	SEM	*p* value
	0 hr	2 hrs	4 hrs	8 hrs	14 hrs	20 hrs			
Magnum									
Fold height (µm)	1125.07	1006.37	1178.31	1306.24	1251.76	1289.83	1192.93	41.82	0.319
Fold width (µm)	733.01	611.39	706.39	555.65	696.76	767.43	678.27	25.74	0.202
Epithelium height (µm)	12.36^{ab}	19.45^{a}	18.45^{ab}	11.93^{ab}	9.68^{b}	19.05^{ab}	15.15	00.96	0.019
Ciliated cells (%)	50.00	49.67	50.40	50.00	50.00	49.80	50.00	00.57	0.999
Secretory cells (%)	50.00	50.33	49.60	50.00	50.00	50.20	50.00	00.57	0.999
Uterus									
Fold height (µm)	1075.84	1324.43	1341.40	977.20	1029.37	1031.58	1126.79	41.00	0.057
Fold width (µm)	179.60	200.51	224.51	194.69	186.88	230.08	203.60	10.15	0.631
Epithelium height (µm)	18.79	20.71	21.83	19.19	20.80	20.11	20.24	00.40	0.293

^{ab} Means with different letters within the same line differ significantly in the Tukey test (p<0.05). SEM: Standard error of the mean.

Table 4 – Serum biochemistry of total protein (g/dL), albumin (g/dL), phosphorus (mg/L), alkaline phosphatase (U/L), total calcium (mg/dL) andionic calcium (mg/dL, calculated by formula) in Japanese quails during the daily egg-laying cycle.

Variable	Time post-oviposition						Means	SEM	*p* value
	0 hr	2 hrs	4 hrs	8 hrs	14 hrs	20 hrs			
Protein	4.77^{b}	5.61^{ab}	5.43^{ab}	4.90^{ab}	4.65^{b}	4.72^{b}	5.01	0.078	0.002
Albumin	1.22^{b}	1.65^{a}	1.42^{ab}	1.33^{b}	1.46^{ab}	1.42^{ab}	1.42	0.027	0.001
Alkaline phosphatase	1202.89	1311.98	1488.06	1384.58	1280.45	1237.86	1316.87	55.592	0.702
Phosphorus	9.26	8.26	8.80	8.69	8.82	8.59	8.74	0.303	0.963
Total Ca	25.56^{abc}	27.98^{a}	26.73^{abc}	26.20^{abc}	25.37^{b}	24.96^{bc}	26.15	0.316	0.037
Ionic Ca	18.76	19.17	18.88	18.91	18.16	17.94	18.65	0.215	0.537

^{abc} Means with different letters within the same column differ significantly in the Tukey test (p<0.05). SEM: Standard error of the mean.

PAS+ (Figure 4B), and AB+ (Figure 3A). After this period (4 hrs POV), the magnum glands were partially emptying, and clearer areas were visible in the PAS stain (Figure 4C). Tubular glands had fewer granules in the cytoplasm and allows secretion content. In group 20 hrs and 0 hr POV, the secretory volume of the magnum increased again, with glandular secretion stocked inside the lumen and cytoplasm of gland cells and the secretory cells of the epithelium of mucosa.

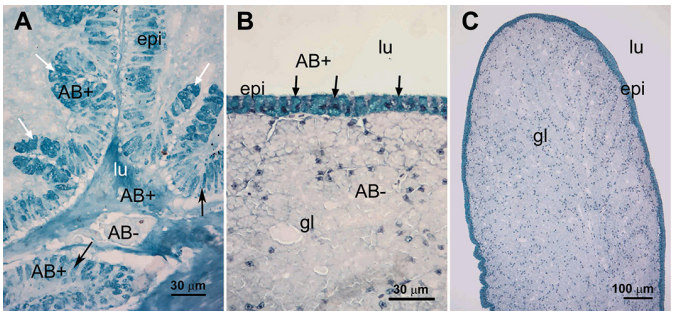

Figure 3 – Photomicrography of histochemical characteristics of magnum in Japanese quails stained with Alcian Blue (AB) pH 2.5. A) The image represents the 2 hrs treatment when the egg is inside the magnum in the albumen secretion phase. In this period, the cytoplasm of the cells of the duct (white arrows) and the secretory cells from epithelium (epi) are filled with strong AB positives granules (black arrows). The content of the granules is released into the lumen (lu) of the magnum, leaving a secretion of albumen AB+ near the surface of the mucosa. B-C) After 20 hrs of oviposition (group 20 hrs), the egg is inside the uterus and the cytoplasm of secretory cells of the epithelium are filled with AB positive granules again (arrows). Note that cells glands (gl), granules, and secretion are AB negative. C showsa low magnification of a fold of the magnum, evidencing the general characteristic of glands with AB negative contents. Scale bar: A-B) 30 µm, C) 100 µm. Paraffin, Alcian Blue pH 2.5.

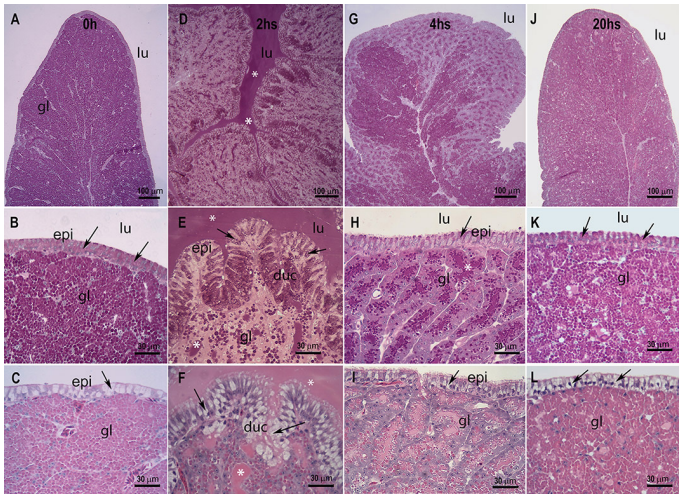

Figure 4 – Histology and histochemical reaction to periodic acid of Schiff (PAS) of the magnum of Japanese quails along the egg-cycle. In general, the cytoplasmatic granules of secretory cells (black arrow) in the epithelium and cells of ducts (duc) are PAS+ and do not stain with HE staining. The tubular glands (gl) of submucosa and secretion inside lumen (lu) have PAS+ and eosinophilic granules (*). A-C) In treatment 0hr, female are ready to ovulate and the magnum is filled with albumen secretion inside the tubular glands of the submucosa, and the secretory cells of the epithelium. D-F) In group 2 hrs POV, the epithelium of the ducts and the lumen of the glands have intense secretory activity, and albumen secretion is observed into the lumen. Note the reduction in cell height in the submucosa glands in high magnification. G-I) In the 4 hrs group, the partial emptying of the tubular glands can be easily visualized in low magnification of the folds. In high magnification, the quantity of granules in cytoplasm continues to be reduced. J-L) For females from the 20 hrs group, the folds are filled again and submucosa glands are empty of PAS+ and eosinophilic granules. The secretory cells also show this effect. Stain: HE+PAS: A-B, G-H, J-K; PAS: D-E; HE: C, F, I, L. Scale bar: 100 µm (A, D, G, and J); 30 µm (B, C, E, F, H, I, K, and L)

The histomorphometry of the uterus was not influenced by egg-cycle period (p>0.05) (Table 3). Histologically, the uterus had ramified folds with 1.13 mm in height and 0.20 mm in width, covered by an epithelium with a height of 20.24 µm, composed by ciliated and secretory cells. The secretory cells had PAS+ and AB+ granules, and uterine glands of submucosa showed eosinophilic, PAS-, and AB+ granules (Figure 5).

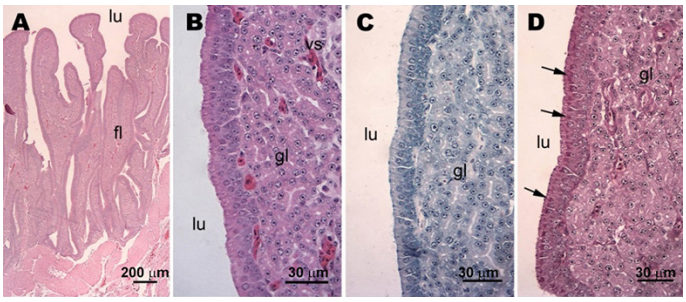

Figure 5 – Uterus photomicrography in Japanese quails in HE (A-B), AB pH 2.5 stain (C) and PAS (D) stain in the 20 hrs group. The branched character of the uterine folds (fl) was evident. The epithelium of the mucosa shows secretory and ciliated cells (arrows) PAS and AB+. The submucosa glands (gl) had eosinophilic, AB-, and weak PAS+ cytoplasmatic granules. Scale bar: A) 200 µm, B-D) 30 µm.

SEM of eggshell

Eggshell of eggs inside the analyzed uteruses showed that the beginning of mammillary body formation occurred 4 hrs POV (around 20h00), while still evidencing the membrane fibers (Figure 6). The final formation of the mammillary layer and the beginning of the palisade layer is already evident 8 hrs POV. In this stage, the mammillary bodies grew to form columns resulting in the palisade layer. Among those columns, gas exchange pores are formed randomly, being characterized by spaces through the entire thickness of the eggshell. In higher magnification, the

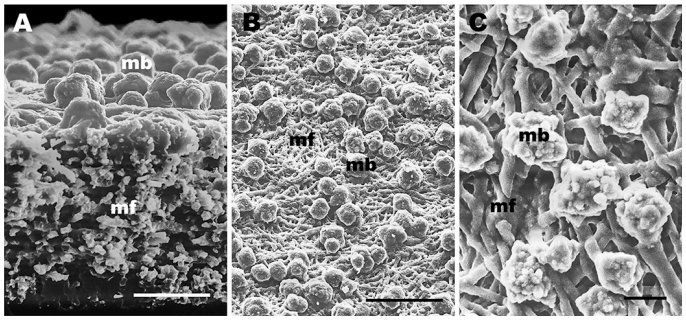

Figure 6 – Scanning electron microscopy in eggshell at 4 hrs POV in cross-section (A) and outer (C-D) views. Notice the beginning of eggshell formation. The mammillary bodies (mb) are rounded formations arranged randomly on the outer shell membrane fibers (mf), which is still visible at this stage of shell formation. Scale bar: A) 20 µm; B) 50 µm; C) 10 µm.

columns of the palisade layer are composed of calcium carbonate crystals growing in aridge display, forming consecutive layers (Figure 7).

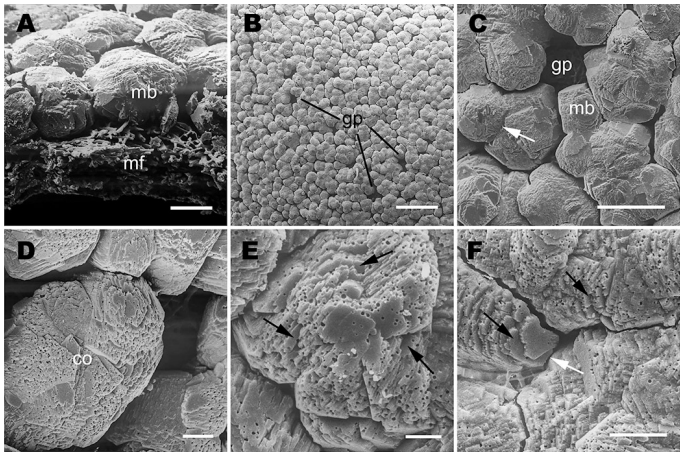

Figure 7 – Scanning electron microscopy in eggshell at 8 hrs POV in cross-section (A) and outer (B-F) views. At this stage, was possible to observe several stages of eggshell formation. The mammillary bodies (mb) were well developed and fully cover the outer shell membrane (mf) from external vision. The growth (D) of columns (co) through the successive deposition (E) of layers of crystals (black arrows) is visible, and later their junction boundary (C, F) (white arrows) forming random spaces characterizing the gas pores (gp). Scale bar: A) 20 µm, B) 200 µm, C) 50 µm, D-F) 10 µm.

At 14 hrs POV, the palisade layer is evident. The outer view shows the irregular, spongy appearance of the surface and it is possible to observe projections of the columns that make up this layer. The junction of these columns results in the uniformity of the layer, which is only interrupted by the gas exchange pores (Figure 8).

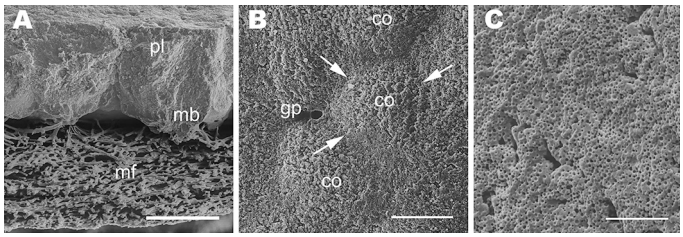

Figure 8 – Scanning electron microscopy in the eggshell at 14 hrs POV in cross-section view (A) and outer (B-C) views. At this stage of eggshell formation, the total junction boundary (white arrows) among columns (co) that were still growing was observed (B), forming the intermediate (A) of the palisade layer (pl) above the mammillary bodies (mb). The outer view of the shell has an irregular spongy appearance (C) and gas pores (gp). Scale bar: A) 50 µm, B) 20 µm, C) 10 µm.

At 20 hrs POV, gas exchange pores and all layers can be identified (mammillary, palisade, and cuticle). The cuticle is the last layer and confers a smoother surface appearance (Figure 9). Only two quails (2/10) had a pigmented eggshell in the 20 hrs POV treatment, one of them with the cuticle, and another one without fixation of the pigment (Figure 2).

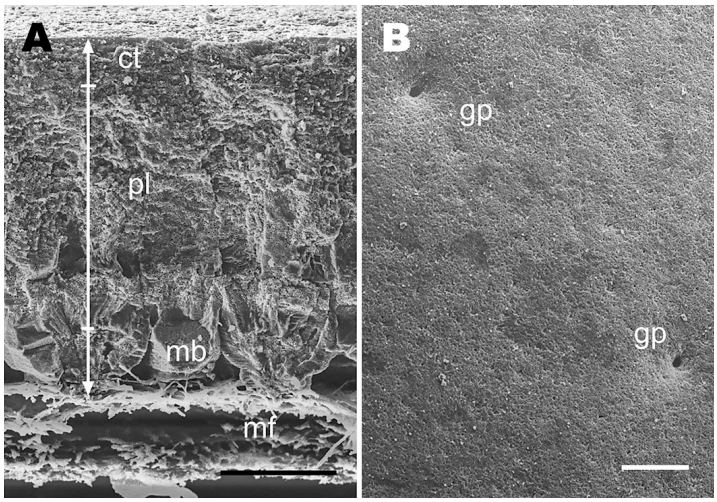

Figure 9 – Scanning electron microscopy in eggshell at 20 hrs post-oviposition in cross-section view (A) and outer (B) views. In this final phase, it was possible to observe all the layers of eggshell formation (A), with distinction of the fibers membrane (mf), mammillary bodies (mb), palisade layer (pl), and the cuticle, which this final layer that gives a smooth appearance to the shell (B), where the presence of sparse gas pores (gp) could be easily distinguished. Scale bar: A) 50 µm, B) 100 µm.

Serum biochemistry

The time of POV influenced the protein, albumin, and total Ca in quail hens (Table 4). Serum protein was higher in the 2, 4, and 8 hrs POV groups; while albumin and total Ca had higher values for the 2 hrs POV quails. There were no significant differences in the serum variables phosphorus, alkaline phosphatase, and ionic calcium.

Table 5 – Bone variables of the femur and tibiotarsus of Japanese quails during the daily egg-laying cycle.

Variables	Time post-oviposition						Means	SEM	*p value*
	0 hr	2 hrs	4 hrs	8 hrs	14 hrs	20 hrs			
Femur									
Hardness (kgf)	2.61	2.97	2.69	2.15	3.14	2.46	2.66	0.11	0.144
Fractureness (kg)	2.49	2.85	2.39	2.13	3.12	2.44	2.56	0.13	0.269
Time (s)	7.20	8.08	7.47	6.18	7.25	5.84	6.97	0.26	0.136
Density (mmAl)	0.99^{abc}	0.35^{c}	0.57^{abc}	0.71^{abc}	1.06^{a}	0.99^{ab}	0.79	0.07	0.012
Seedor (mg/mm)	14.43	14.56	15.23	15.16	14.29	14.82	14.77	0.28	0.863
Tibiotarsus									
Hardness (kgf)	2.51	2.57	2.37	2.47	2.34	2.55	2.47	0,08	0.948
Fractureness (kg)	2.37	2.57	2.24	2.47	2.34	2.51	2.42	0.09	0.088
Time (s)	7.56	10.42	8.54	10.66	9.18	10.50	9.65	0.31	0.064
Density (mmAl)	0.92^{ab}	0.43^{b}	0.51^{ab}	0.61^{ab}	1.02^{a}	0.84^{ab}	0.73	0.06	0.039
Seedor (mg/mm)	11.99	12.12	12.74	11.52	11.73	11.34	11.90	0.20	0.390

^{ab} Means with different letters within the same line differ significantly in the Tukey test (p<0.05). SEM: Standard error of the mean.

Bone analysis

The bone variables of the femur and the tibiotarsus (hardness, fractureness, time of the first break, and Seedor index) were not influenced by day egg-cycle period ($p>0.05$) (Table 5). It was found that the femur resists up to an average of 2.65-kg for 7 seconds before fracturing, which represents 16 times its weight. The tibiotarsus bone resists for an average of 2.42-kg for 9.7 seconds before breaking, 14.40 times its weight. Bone density measured by the radiograph with a penetrometer showed the highest average in the 14 hrs and the lowest average in the 2 hrs POV for the femur and tibiotarsus.

Total bone weight or the relative weight of cortical and medullar bone, and the percentage of ashes in these portions in the femur and tibiotarsus were not influenced by the day egg-cycle in quails. Femur had a mean weight of 0.54 g (0.42 cortical and 0.12-g medullar bone) and the tibiotarsus had a mean weight of 0.55 g (0.44 cortical and 0.11-g medullar bone) (Table 6). The Ca and Pin the medullary bone of the femur were influenced by POV, with the highest values for 0 hr POV (Ca 16.43; P 8.15), and the lowest values in the 14 hrs POV (Ca 9.42; P 4.35).

The Ca content of the medullary bone was significantly higher in the 0 hr POV group, and lower in the 14 hrs POV one for both bones. The concentration of Pin the medullar bone in the femur, and in cortical bone in the tibiotarsus showed a significant difference among the treatments (Table 6).

DISCUSSION

This research described the morphological changes during the egg-cycle in Japanese quails in the ovary, magnum, uterus, femur, and tibiotarsus. Eggshell deposition was also described along the analyzed periods.

Analysis of the relative weight of ovary + oviduct, and follicular diameter show that the increase in weight during the cycle of formation of the egg was a function of the increase in the weight of pre-ovulatory follicles that accumulate vitelline content and consequently increase in size. The mean values found in this study of follicular diameter are similar to those described by Sreesujatha *et al.* (2016) in quails, suggesting a follicular growth pattern.

After the ovulation of the largest follicle (F1), the preovulatory yellow follicle grows by increasing yolk content. The F2 moves in the hierarchy to be F1 and so on, so that the largest follicle will be the next to be ovulated and the others increase in volume afterwards. The weight and diameter of the ovarian follicles followed this hierarchical behavior in the ovary of the analyzed quails along the groups. In hens,

Table 6 – Means of weight, ashes, calcium (Ca), phosphorus (P) and proportions in cortical and medullary bones in femur and tibiotarsus of Japanese quails during the daily egg-laying cycle.

Variables	Time post-oviposition						Means	SEM	*p value*
	0 hr	2 hrs	4 hrs	8 hrs	14 hrs	20 hrs			
Femur									
Weight (g)	0.52	0.57	0.56	0.55	0.52	0.52	0.540	0.01	0.480
Cortical (%)	80.60	79.99	76.31	77.69	79.25	76.99	78.42	0.60	0.269
Medullar (%)	19.40	20.01	23.69	22.31	20.75	23.01	21.58	0.60	0.269
Ash cortical (%)	67.01	63.65	63.53	63.63	61.54	64.90	64.06	0.80	0.516
Ash medullar (%)	56.09	52.17	42.09	46.38	40.47	44.68	46.80	1.91	0.184
Ca cortical (%)	12.00	12.21	13.34	11.81	10.71	9.38	11.55	0.85	0.812
Ca medullar (%)	16.43^{a}	13.48^{ab}	11.83^{ab}	11.48^{ab}	9.42^{b}	13.20^{ab}	12.61	0.57	0.034
P cortical (%)	8.09	7.23	7.91	7.73	7.78	7.42	07.71	0.52	0.997
P medullar (%)	8.15^{a}	6.92^{ab}	6.27^{ab}	5.60^{ab}	4.35^{b}	7.45^{a}	06.44	0.29	0.010
Tibiotarsus									
Weight (g)	0.55	0.56	0.59	0.53	0.55	0.52	00.55	0.01	0.359
Cortical (%)	82.81	79.49	81.23	77.82	81.28	80.59	80.54	0.50	0.117
Medullar (%)	17.19	20.51	18.77	22.18	18.72	19.41	19.46	0.50	0.117
Ash cortical (%)	72.22	70.42	69.54	73.21	69.89	70.29	70.93	0.55	0.346
Ash medular (%)	50.79	47.53	45.50	46.44	42.07	44.13	46.08	1.18	0.406
Ca cortical (%)	16.93	16.14	15.86	15.41	17.29	17.97	16.63	0.71	0.903
Ca medullar (%)	13.30^{a}	9.92^{ab}	11.83^{ab}	9.22^{ab}	6.45^{b}	10.41^{ab}	10.20	0.57	0.036
P cortical (%)	9.77^{ab}	8.06^{b}	9.78^{ab}	9.87^{ab}	11.86^{a}	11.48^{a}	10.08	0.32	0.032
P medullar (%)	7.56	6.87	5.90	7.27	5.19	6.20	06.50	0.26	0.124

^{ab} Means with different letters within the same line differ significantly in the Tukey test ($p<0.05$). SEM: Standard error of the mean.

prehierarchical follicles can remain at rest for months, entering the hierarchy when they reach 6 to 8 mm, after which only one follicle develops and ovulates in 5 to 10 days (Hrabia, 2021).

The general weight of the different segments of the oviduct in this experiment was similar to that described by Turner & Eliel (1978), who obtained mean weights of the magnum, isthmus, and uterus, of 3.26, 0.84, and 2.02 g, respectively. The magnum presented higher relative weight in the periods of 0 and 20 hrs POV, evidencing that in these periods the magnum was being prepared for developing the next egg, which leads to an increment in its weight as the oocyte moves along the oviduct toward the magnum. Perhaps this increase in weight is also related to the increase in the circulatory supply of the muscular layer to increase the contraction that promotes the passage of the egg and spermatozoa.

In this study, the magnum folds had a height of 1.19 mm and a width of 0.67 mm. Its mucosa presented large folds, broad and visible macroscopically, as compared to another region of the oviduct. The histological results confirmed that the magnum of quails did not modify the cellular proportion of ciliated and secretory cells (1:1) during the egg cycle. In hens, the magnum changes the cell type of the oviduct magnum in an age-specific manner, and an old female will have a different pattern of cells (González-Morán, 2016).

The size and shape of the folds of the magnum were in agreement with the previous reports for quails (Artoni *et al.*, 2001; Moraes *et al.,* 2007; Carneiro *et al.*, 2014). Although the studies for quails are not recent, this species did not undergo the same genetic selection that happened to chickens over time, so that these data can be compared even with the current quails in this experiment.

Although there were no changes in fold size during the different stages of egg formation, physiologically relevant changes in the secretory activity were observed. In general, avian eggs remains in the magnum for about 3 hrs, resulting in changes related to the passage of the egg, providing a series of physiological events that promote the release of albumen (Jacob & Bakst, 2007).

As shown in this work, the magnum has numerous tubular glands with eosinophilic cytoplasm and PAS+ granules. In avian reproduction, egg white or albumen provides essential nutrients as well as protection against invading bacteria for embryo development (Hu *et al.*, 2016). The tubular glands of the magnum are responsible for the production of albumen, especially proteins like ovalbumin, lysozyme, ovotransferrin, conalbumin, and antimicrobial peptides, primarily under the stimulatory control of estrogen (Socha & Hrabia, 2018). Artoni *et al.* (2001) observed that high protein levels in food increased glandular layer thickness in quails, suggesting that protein from the diet can affect egg quality.

The strong eosinophilic staining and histochemical PAS+ reaction in glandular secretion observed in this research indicate that the albumen composition of quails has a similar chemical composition to that of other avian females (Parizzi *et al.*, 2008; Apperson *et al.*, 2017). Recent research described a closer relationship between quail and chicken egg white proteome patterns, but with specific proteins in quails. This suggests that even belonging to the same Galliformes order, *Gallus* and quail developed unique needs for egg white protein functions during their long-term evolution (Hu *et al.*, 2016). Then, not only the time of oviposition is distinct in these two species, but also specific points can be addressed.

We qualitatively observed that the cytoplasm of the tubular glands in the magnum was full of granules in females from groups at midday (20 hrs POV) and after laying eggs (0 hr POV). Although the presence of albumen secretion was constant, the production and secretion were more intense in quails from group 2 hrs POV (around 18h00), after ovulation, and during the passage of the egg through the magnum. This is supported by authors describing this process in hens. The peak of secretion occurred at the time of ovulation when there was the greatest amount of secretion in the tubular glands (0 hr POV group). Quails in those groups were analyzed between 16h00 and 18h00 when the day is finishing and the light program will have more than 4 hrs of light, while hens in the same egg-cycle period will be in the morning and have more than 8–10 hours of light.

Regarding serum biochemistry results, higher serum protein levels corresponded to periods of albumen production when the magnum is in the active secretion phase, i.e. groups 2 and 4 hrs. Serum proteins are central to the formation of albumen. Albumin, with globulin, forms a fraction of total plasma proteins (Schmidt *et al.*, 2007), so it presented similar behavior, suggesting that secretion and albumen production are constant, being larger or smaller according to the position in which the follicle is in the oviduct. Blood levels of protein and albumin are higher in females than males, like total cholesterol and triglycerides. These parameters are ingredients in egg, albumen, and yolk formation (Scholtz *et al.*, 2009; Agina *et al.*, 2017).

The macro and microscopic aspects of the uterus were in agreement with the general avian descriptions. The morphology and histochemistry of the epithelium of the uterine mucosa were also in agreement with the previous citations for quails (Moraes *et al.*, 2007; Moraes *et al.*, 2009). The uterus of quails is similar to that of laying hens, except for the dark-brown coloration of the mucosa, as described by Artoni *et al.* (2001). Carneiro *et al.* (2014) observed mean height and width values for the uterine folds of 1.26 and 0.26 mm, respectively, compared to data found in this study.

Albumen hydration can be observed in this research in the egg weight increment from groups 4 hrs to 8 hrs POV. In these same groups, eggshell was just in firsts stages of deposition, while weight increased by 30%. The uterus causes albumen hydration by plumping fluid and swelling the egg, causing a reduction in albumen protein and an exposition of mammillary bodies that have already been deposited on the egg membranes (Solomon, 1991). In hens, 15 g of fluid and ions (Na, K and Cl) are added in this process, at high levels in first hours, but reducing after eggshell calcification.

In quails, fluctuation in phosphorus and ionic Ca was not evidenced. The volume of minerals obtained in bone reabsorption during the dark hours was probably not sufficient to affect blood levels, and hormones of Ca and P metabolism (e.g. parathyroid hormone and 1,25-dihidroxyvitamin D_3) maintained homeostasis.

Shell formation is a biomineralization process based in the secretion of ionic calcium from the blood to the uterine lumen. Carbonic anhydrase enzyme catalyzes Ca and bicarbonate in the tubular glands of the uterus to an amorphous phase, turning them into calcium carbonate or calcite. Calcite is considered a natural and porous ceramic, and is deposited in layers on the membrane of the shell, forming the mineral base of the eggshell (Hincke *et al.*, 2012; Holm *et al.*, 2001; Nys *et al.*, 2021).

These mineral layers and porous aspect were also evident in eggshell SEM observation in samples analyzed from eggs in quails in the early morning (14 hrs POV) and midday (20 hrs POV) groups. Shell structure is similar in different bird species, and calcite is the common mineral base in avian families (Solomon, 1991).

The influx of Ca into the uterus to form eggshell creates a circadian rhythm as a function of the ovulation stage and eggshell formation (Nys *et al.*, 2021). During periods of light, the laying hen, through ingestion of Ca from the diet, keeps her body in homeostasis. Until 6–9 hours after oviposition, the serum concentration of ionic calcium increases and phosphorous decreases. In hens, between 8- and 20-hours post-ovulation, the plasma concentration of ionic Ca decreases, and bone medullary Ca is reabsorbed and transported to the uterus to form calcium carbonate for shell mineralization (Kerschnitzki *et al.*, 2014). According to the same authors, as phosphorus is not required for the formation of calcium carbonate, its plasma concentration increases, reaching a peak around 12-h post-ovulation. Clunies *et al.* (1993) analyzed activity with labeled Ca and obtained average values of total blood Ca similar to our work in quails, which are stable during the cycle of egg formation.

In this research, the light program turns lights off at 22h30, and eggshells analyzed in the group 8 hrs POV (24h00) had only mammillary bodies secreted on the shell membranes. Lights turns on again at 05h30 in the morning and eggshell had only few portions of palisade layers in the group sampled at 06h00 (14 hrs POV), which indicates that a major part of eggshells in quails is secreted in the following hours, when quails have access to food and Ca sources from the diet.

Calcium for eggshell formation is endogenous, about 2/3 comes from the diet, complementing the Ca available from the medullary bone (Kim *et al.*, 2012; Ribeiro *et al.*, 2016). A synchrony between intestinal Ca absorption increments and uterine requirements for shell formation is observed in hens (Clunies *et al.*, 1993). After the start of egg calcification (active phase), the calcium used to form the shell comes from the reabsorption of the medullary bone (Kerschnitzki *et al.*, 2014).

In quails, metabolism and bone dynamics were described as similar to that of laying hens (Taylor & Dacke, 1984), but with a singularity in Ca metabolism, since most of the shell calcification occurs during the day, whereby dietary intake and calcium absorption by the intestine occur intensely, not using the medullary bone as a source of calcium (Bar, 2009).

Our results in serum ionic Ca and bones reinforce these descriptions. The results of bone analysis in this research with quails demonstrated that bone strength did not change during egg formation, even in the shell formation phase by depositing calcium carbonate in periods 4 to 20 hrs POV. The general characteristics of the bone observed in our work did not change with the absorption and reabsorption of daily Ca, necessary for the mobilization of the Ca used for the formation of the eggshell, the density and mineral concentration of Ca being more reliable parameters for the evaluation of daily Ca mobilization.

Medullary bone is a fast-turnover structure, with numerous osteoclasts on its surface, and osteoclastic activity in hens' bones can offer about 30-40% of the Ca in the shell and can be metabolized at a rate that is 10 to 15 times faster than that of cortical bones (Van De Velde *et al.*, 1984; 1985). Their destruction and reconstruction are extremely fast and, in cases of Ca deficiency, at the expense of the cortical bone. Its total volume did not change during the poultry laying cycle, only its degree of calcification (Dacke *et al.*, 1993). In our results for the femur and tibiotarsus, weight of bones, and relative weight or ashes (%) of cortical or medullar bones were not influenced by the period of day.

After oviposition, the osteoblasts replace the osteoclasts and regenerate the medullary bone. Although medullary bone is important for shell formation, there is no direct relation between the shell and bone quality (Whitehead, 2004). In the 6–9 hours after oviposition period, an increase in medullary bone mineralization in hens occurs, but there is no change in trabecular bone thickness (Kerschnitzki *et al.*, 2014). After the onset of calcification of the next egg, a decrease in the mineral content of the medullary bone is observed, with the presence of numerous active osteoclasts (Van de Velde *et al.*, 1985; Rodriguez-Navarro *et al.*, 2018).

Mineral content dynamics of the medullary bone through periods during the daily cycle of egg formation were also observed in quails in this experiment. The reduction of Ca% in the ashes of the medullary bone of the femur and tibiotarsus analyzed in females 14 hrs POV suggested that, during the previous hours, medullary bones were probably the main source of minerals for the formation of the eggshell. In quails with the evening laying behavior, this comprises the dark hours of the light program (22h30 to 05h30). During this period, around 25% of eggshell thickness was deposited, as evidenced by the scanning electron microscopy results.

In laying hens, eggshell formation occurs mainly during the night, when birds stay in the dark and are not eating. In the evening, when there is no calcium offering, there is bone mobilization, enough to avoid bad calcification of the shell. In this sense, blood biochemistry and bone results in quails strongly suggest that they have a notably different egg cycle metabolism, spending most of the time on eggshell secretion while having light and, consequently, diet and Ca feeding. Therefore, we believe that the Ca source from medullar bone may have less importance in quails than in hens. On the other hand, diet in the morning is essential to a good eggshell deposition.

Additionally, based on our results, when the aim is to get samples for experiments on quails during laying production, the physiological period of egg formation should be considered to avoid bias. Samples obtained in the afternoon, and particularly in the late afternoon, can be influenced by physiological variations of the daily egg-cycle and affect the obtained results. Thus, it is strongly recommended that researchers collect blood, bone, or genital organ samples in quails between 6 and 12 am to avoid bias.

Finally, the oviduct of Japanese quails presented morphological variations as a function of the period of egg formation, as well as the blood concentration of Ca and the Ca content of the medullar bone of the femur and tibiotarsus bones, maintaining homeostasis in each phase studied of the daily egg-laying cycle.

CONCLUSION

In conclusion, the eggshell secretion in light periods, the high magnum activity, and the medullary bone Ca deposition during midday and afternoon as well as the ovulation/oviposition in the afternoon are the main distinct characteristics of the physiological aspects of the egg-cycle in quails.

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