A histological study of post-mortem specimens taken from dead-in-shell ostrich (*Struthio camelus*) embryos

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SUMMARY

The current study investigated the histology of various thoraco-visceral tissues of 85 dead-in-shell ostrich embryos. The average embryo length was 16.3 cm and weight 734.2 g. The average egg dimensions were: a) Smaller class - 1,560 g weight, 155.1 cm length, and 128.4 cm width; and b) Larger class - 1,592 g weight, 156.1 cm length, and 129.5 cm width. There were visible numerous lymphocytic infiltrations in the kidney cortex. No patho-morphological alterations were observed in the trachea or tongue. The gizzard had lymphocytic infiltrations and myopathy. The heart muscle showed anasarca and myopathy. We suggest a genetic element possibly associated with a nutritional imbalance and a microbial spoilage of eggs that is predisposing to the dead-in-shell embryos on the farm.

Key words: histology, viscera, ostrich embryo, microbial spoilage

INTRODUCTION

Ostrich hens reach sexual maturity at approximately 2.5 years and remain fertile for about 40 years. Each hen commonly lays about 50 eggs a season. Selective breeding, good breeder nutrition, hygiene and correct incubator settings are important for good productivity (Cooper, 2001). Unbalanced breeder nutrition may result in multifaceted nutrient deficiencies in embryos including yolk sac pathology (Cooper and Horbańczuk, 2004), micrognathia (Perelman *et al*., 2001) and leg abnormalities (Perelman *et al*., 2001; Horbańczuk *et al*., 2004). Another study in conjoined ostrich twins demonstrated that the gastro-enteric apparatuses were not completely developed and fused at different levels, although they shared a common liver and heart (Mazzullo *et al*., 2007). Ostrich twinning from a single- (Horbańczuk *et al*., 2001) and double-yolked (Horbańczuk *et al*., 2003) egg has been described.

Malpositioning and severe oedema are major symptoms of dead-in-shell embryos, with myopathy, gross lesions of viscera, haemorrhage, bacteriology and congenital deformities playing a minor role (Brown *et al*., 1996). Microbial contamination is a significant factor in embryonic death (Deeming, 1995). A published study on ostrich embryos determined the multi-factorial effects of inappropriate incubator settings (high temperature and low oxygen partial pressure), unbalanced nutrition in

the hens, infection or genetic problems associated with the expression of lethal genes (Cooper and Horbańczuk, 2005).

Currently there is a dearth of papers investigating the histological aspects and pathology of ostrich embryo development. Therefore, the aim of the current study was to examine and report on the histology of various thoraco-visceral tissues of dead-in-shell ostrich embryos.

MATERIALS AND METHODS

Eighty-five dead-in-shell ostrich embryos laid by hens aged 18 months housed on an ostrich farm in Poland (Ferma Strusi Stypułów, 67-120 Kożuchów; 51º43´N and 15º33´E; October 2007) were obtained. The stage of embryonic mortality ranged between Stages 6-7 according to standard candling charts (Cooper, 2001). On candling Stage 6 shows an air cell boundary that is dark and clearly-defined with the embryo shadow occupying approximately two-thirds of the egg (Cooper, 2001). Stage 7 involves a very dark embryo shadow occupying approximately seven-eighths of the egg. The ambient indoor and exterior temperature from 0600-1800 was recorded at 2-hr intervals as 20.40 (mean) ± 0.12 (SEM) and 14.98 ± 1.35 ºC, respectively. The exterior relative humidity recorded likewise was 50.00 ± 0.67%. A maximum time period for incubation of 45.9 days with an interval of 12.7 hours between pipping and hatching was allowed (Deeming, 1995). Measurements of length, width, horizontal and vertical circumferences, and egg weight were recorded. The presence of an embryo shadow in an unhatched egg prompted isolation for further study. Those without a shadow were not used. Eggs were opened manually using a hammer. The albumen was carefully poured out and the embryos removed and placed on paper towel. The length of each embryo from the dorsal aspect of the head to the tip of its distal abdomen, and body weights (minus the yolk sac) were recorded. Following an incision ventrally from the undeveloped beak to the cloaca, tissues from the tongue, trachea, gizzard, caecum, liver, rectum, heart, lungs and kidneys were excised and placed in 10% formaldehyde contained in 5 mL Eppendorf tubes.

Tissues were fixed in 10% formaldehyde for 24 hr. Thereafter tissues were washed in tap water for 2 hr, and processed in a tissue processor [60% (1hr), 70% (2hr), 80% (2hr), 96% (2hr), and 96% (3hr) ethanol; absolute ethanol (3hr); xylene I (2hr) and II (2 hr); and paraffin I (2hr) and II (2hr)] (Tissue-Tek II, Sakura, Japan). Paraffin-embedded tissues were sectioned 4 µm thick on a microtome (SLEE CUT-4055, Technik GMBH, Germany) and stained. Stains were prepared according to well-established methods described in Carson (Carson, 1997). Standard Haemotoxylin and Eosin (H & E) staining involved deparaffinising and hydrating slides in distilled water, staining, dehydrating with 95% and absolute ethanol, clearing with xylene and mounting in synthetic resin (Tissue- Mount, Sakura, Japan).

Photos of histological slides were taken with a microscope Olympus BX 41 (Berlin, Germany). The minimum scale of 1 µm was chosen. The optics in the infinity correction system was of the UIS-2 type, and the optical length of lens was 45 mm. The built-in light strength regulator with a constant power lighting switch was used in microphotography. Glasses with rubber guards/pads, 10 × enlargements and area/field number 22 were set. The universal plan achromatic objectives were used with an enlargement of the minimum working distance (WD) of 10 × 0.25/WD & 10.6 mm, and 40 × 0.65/WD & 0.6 mm.

A digital colour camera was used to take the images with an ArtCam 300Mi and a built-in matrix of CMOS type with the resolution of 3 megapixels, connected to a computer with USB 2.0. Quick Photo Camera 2.2 (Microsoft 2007) was used to determine image resolution and measurement of object sizes interactively. An ISO certificate 14001/2004, and 9001/2000 and declaration of conformity were noted. Confirmation of histological diagnosis was confirmed during consultation with a registered anatomical histopathologist.

All experiments were performed in accordance with the guidelines of the Animal Ethics Committee for Animal Research, Poland (49/05/04 and 106/06) under the auspices of EU Scientific Standards.

RESULTS

The average embryo length was 16.3 cm and weight 734.2 g. There were no apparent visual observations of abnormal embryonic positioning or of any morphological and anatomical malformations. All embryos were mostly feathered with complete ventral downiness. The average egg dimensions were: a) Smaller class - 1,560 g weight, 155.1 cm length, and 128.4 cm width; and b) Larger class - 1,592 g weight, 156.1 cm length, and 129.5 cm width, respectively. The incubator temperature on collection was 36.2 ºC and the relative humidity 25%.

Histopathological observations

Kidney - The renal cortex was composed of a visible Malpighian tubule system made of glomerulus surrounded by the Bowman's capsules. There were visible numerous lymphocytic infiltrations (Figure 1).

Trachea - The photo-micrographs showed a longitudinal cross-section through the trachea wall. Visible hyaline cartilage rings of regular structure infiltrated with chondrocytes in cartilaginous lacunae were noted (Figure 2a). The transverse section through the embryo tongue (Figure 2b) also demonstrated lymphocytic infiltrations. There was no evident disease of the connective tissue, longitudinal muscular coat and circular muscular coat. No patho-morphological alterations were observed.

Gizzard - The cross-section through the gizzard showed that there was visible fatty tissue with lymphocytic infiltrations and visible connective tissue bands between muscle fibres (Figure 3).

Heart - The heart muscle presented with karyopathy traits and there were evident oedema and lymphocytic infiltrations (Figure 4). There were subtle degenerative changes in the musculature and fibrinoid degeneration of arterioles.

Lung - The cross-section of the lung parenchyma showed small cystic ductules and pulmonary alveoli that were undeveloped (Figure 5). There was no evident parenchymal degeneration.

Liver - The liver has a compact structure, although the borders between the hepatic lobules were not clear and in places, blurred. There was a high density of erythrocytes, possibly suggesting hyperaemia of the tissue (Figure 6) or inadequate washing prior to fixing. There was no evidence of hepatitis or fatty deposits.

Caecum - There were lymphocytic infiltrations in submucosa/submucous membrane to a small extent, and the wall of the caecum did not show villi, but rather, deep crypts (Figure 7).

Rectum - The mucous membrane was convoluted into oblong folds, containing bands of smooth muscle. There were lytic changes within the tissue with patho-morphological alterations of mucosal glands (Figure 8).

Figure 1. Transverse section of embryo kidney (×40); mt- Malpighian tabulae/ corpusculum renis, l-lymphocytic infiltrations

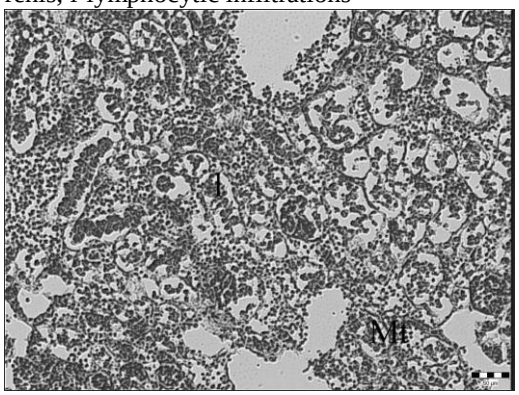


Figure 2A. Transverse section of embryo trachea (×10); lp-lamina propria, f-elastic fibres, h-hyalin cartilage, g-goblet cells

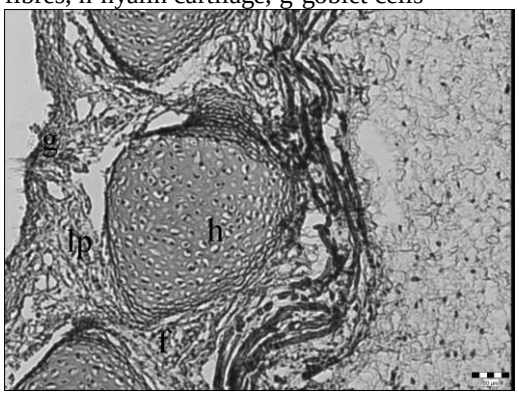


Figure 2B. Transverse section through the tongue (×10); t-connective tissue, ml-longitudinal muscular coat, mc-circular muscular coat, l-lymphocytic infiltrations

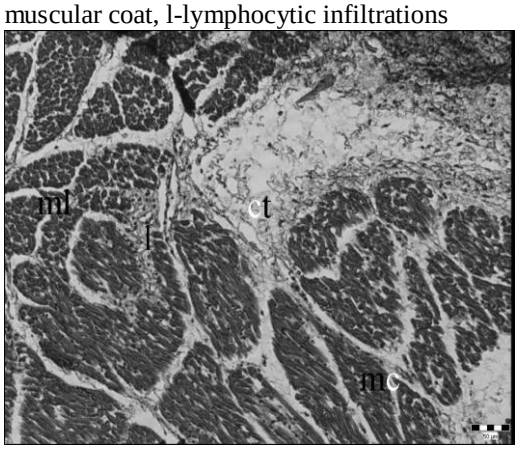


Figure 3. Transverse section of embryo gizzard wall (×10); m-muscular tissue, l-lymphocytic infiltrations

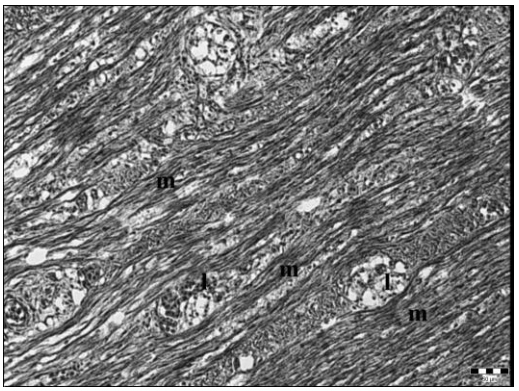


Figure 4. Transverse section of embryo heart muscle with carypathy traits (×10); m-myocardium, o-oedema, l-lymphocytic infiltrations

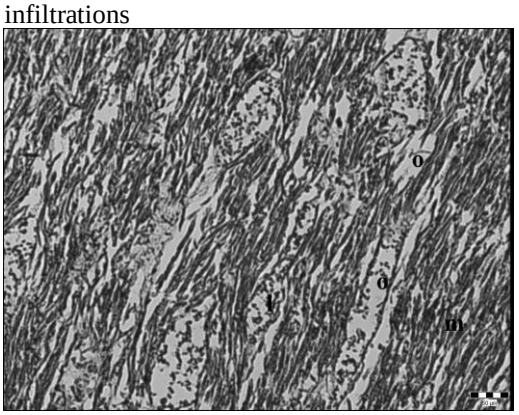


Figure 5. Transverse section of embryo lung (× 10); l-lymphocyte infiltrations, p-pulmonary alveoli lumen, b-bronchi lumen

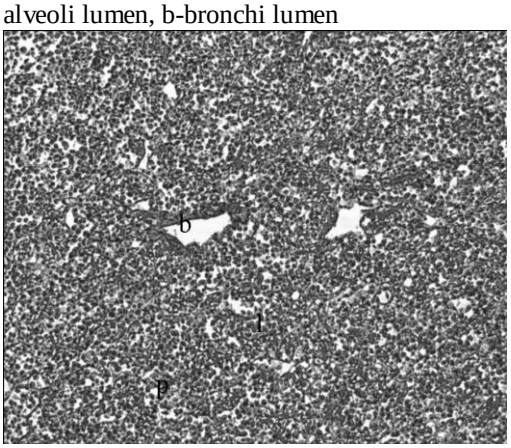


Figure 6. Transverse section of embryo liver (×10); h-hepatocytes, l-lymphocytic infiltrations

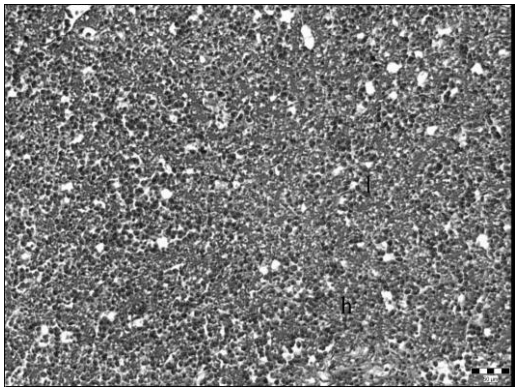


Figure 7. Transverse section of embryo caecum (×10); k-intestinal crypts, l-lymphocytic infiltrations, sm-submucosa/submucous membrane

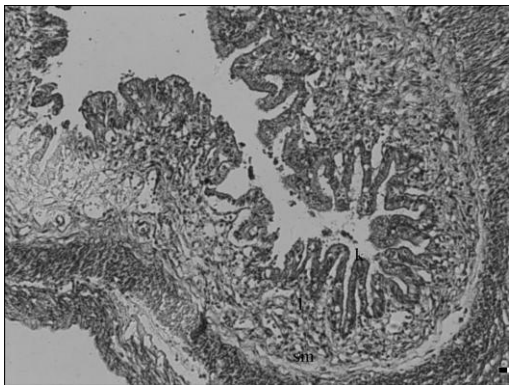
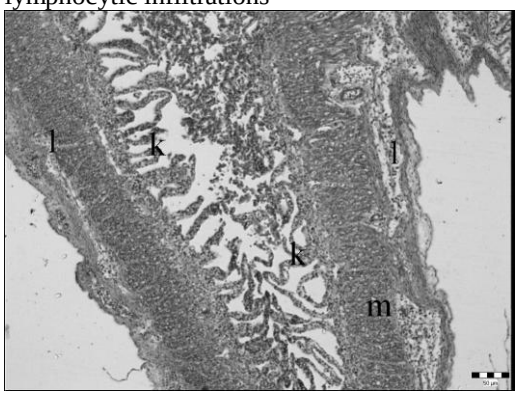


Figure 8. Transverse section of embryo rectum (×10); k-intestinal crypts, m-longitudinal position of the smooth muscle fibres, l-visible lymphocytic infiltrations



DISCUSSION

Egg dimensions were considered as intermediate (>1,450 - ≤1,650 g) and likely to have the best hatchability (Gonzalez *et al.*, 1999). Storage of eggs was 12 days, a time which is regarded as adequate according to Hassan *et al.* (Hassan *et al.*, 2005) who recommended the most effective storage period to be less than 15 days. There was evidence of a holistic patho-morphological effect on organ function, suggesting a multitude of factors responsible for in-shell death.

Localised infection of the kidneys may have been promulgated systemically. The gizzard showed signs of myopathy. Heart pathology was suggestive of anasarca and myopathy, as a consequence of possible embryo malpositioning (Philbey *et al.*, 1991) due to infection. Bacterial infection as a consequence of myopathy was suggested in the caecum. The colon in the ostrich, in conjunction with the coprodeum, is a functional segment for storage and mixing of faeces, although the coprodeum in the ostrich may play a much lesser role in post ureteral modification of urine than is the case in other birds (Skadhauge *et al.*, 2003). However, it functions like a bladder due to the unusually thick layer of mucus (Skadhauge *et al.*, 2003). The role of mucin in establishing a thick unstirred layer required further evaluation (Skadhauge *et al.*, 2003). In our study the mucus membrane presumably contributes ultimately to the production of mucin, although the observed patho-morphology would interfere with this process.

We suggested a genetic element possibly associated with a nutritional imbalance and a microbial spoilage of eggs that is predisposing to the dead-in-shell embryos on the farm. We do not suggest that there is a problem with incubation temperature or relative humidity, nor with hatchability of

fertile eggs which was recorded in the 2007-8 season as 69.3%. Indeed Kennou Sebei and Bergaoui (Kennou Sebei and Bergaoui, 2008) recommend hatchability of 70.07% as acceptable. Additionally, on the farm in the current study the 2007-8 ostrich egg fertility was 68.5%.

Diverse negative effects of some maternal nutritional deficiencies on avian embryos were reported (Cooper and Horbańczuk, 2004; Perelman *et al*., 2001). The action of vitamins, minerals and oligo elements on certain organs and tissues is demonstrated. Selenium and vitamin E deficiency result on lesions involving gastrointestinal smooth muscle in ducks (Yarrington and Whitehair, 1975), alteration of gizzard smooth muscle in ducklings (18), myocardial lesions in turkey poults and ducklings (Ferrans and van Vlet, 1985). Mushi *et al.* (Mushi *et al*., 1998) reported in four adult ostriches developing sudden paresis, low serum selenium and vitamin E levels. After given multi-mineral and vitamin E supplement, no more cases were observed in the remaining birds.

Microbial contamination of eggs represents a serious problem affecting ostrich embryonic development and viability. Deeming (Deeming, 1995; 1996a; 1996b) reported 18- 21%, 22.8% and 32.6%, respectively of eggs spoiled with bacteria and/or fungi. The degree of contamination depends on the environmental hygiene (in the nest, during the storage, in the incubator). For it, the author as well as Mushi et al. (Mushi *et al*., 2008) insists on the necessity of preventive hygienic measures. However, Mushi et al. (Mushi *et al*., 2008) recorded 7.3% of hatchability depression generated by microbial spoilage in spite of the stringent hygienic measures taken by the workers on the farm. The high shell porosity facilitates the entry of germs and the contamination of the egg (Deeming, 1995). We suggest therefore that studies on nest and handling microbiology are completed to determine microbial populations and loads.

The genetic origin of embryonic mortality is demonstrated in several poultry species. Sewalem and Wilhelmson (Sewalem and Wilhelmson, 1999) recorded in the White Leghorn lines selected for egg production traits, a higher mortality at hatch in the selected lines than in the control line. Liptoi et al. (Liptoi *et al*., 2005) showed the genetic effects on the early embryonic mortality and chromosomes abnormalities in three goose lines. Sellier et al. (Sellier *et al*., 2005) observed effects of genotypes on embryonic mortality in common and mule duck eggs incubated for 5 days.

Embryological investigation in the Japanese quail has demonstrated high mortality in the homozygous embryos for *Bh*-gene and many lesions: body haemorrhage, degeneration of liver tissue, less well-developed eyes and occasionally less well-developed limbs and tails (Minezawa and Wakasugi, 1977). Although attempts have been made at developing methods for determining genetic characteristics of ostriches using DNA fingerprinting (Sacharczuk *et al*., 2001) and microsatellites (Kawka *et al*., 2007), and microsatellite markers with chickens (Horbańczuk *et* al., 2007), there is a dearth of specific embryological investigations into the possibility of lethal genes predisposing dead-in-shell manifestations. Hence we advocate further studies on the like.

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