Comparative Histomorphological and Ultrastructural Study of the Luminal Epithelium of the Isthmus in Laying and Moulting Domestic Fowls (*Gallus domesticus*)

M.M. Mpango and M.-C. Madekurozwa*

Department of Anatomy and Physiology, University of Pretoria, Private bag X04, Onderstepoort 0110, South Africa.

*Correspondence:

Tel.: +27 12 529 8417; fax: +27 12 529 8320; e-mail: mary.madekurozwa@up.ac.za

With 11 figures.

Short version of title: Oviduct of domestic fowl

Key words: avian, oviduct, isthmus, moult, apoptosis, autophagy, necrosis, TUNEL

Summary

The present study describes ciliated, non-ciliated and mitochondrial luminal epithelial cells of the isthmus in laying and moulting domestic fowls using histological and ultrastructural techniques. The ciliated cells were non-secretory, while numerous electron dense secretory granules were present in the non-ciliated cells of laying birds. Mitochondrial cells, occurring in two morphologically distinct forms, constituted the third type of epithelial cell present in the isthmus. The SEM study showed that the luminal epithelium was dominated by ciliated cells, the cilia of which partially obscured

adjacent non-ciliated cells. The involution of the luminal epithelium in moulting birds occurred via autophagy, apoptosis and necrosis. Autophagic inclusions, which included autophagosomes and autolysosomes were present in the early degenerative phases of ciliated, non-ciliated and mitochondrial cells. Non-ciliated cells underwent degeneration via apoptosis, which was characterized by nuclear and cytoplasmic condensation. Apoptotic and necrotic ciliated cells were evident during the intermediate and advanced stages of regression. The presence of apoptotic cell death was confirmed using the TUNEL assay. Loss of cilia via the formation of cilia packets was observed using TEM and SEM. Necrotic cell death occurred in mitochondrial cells during the intermediate and late stages of degeneration.

In conclusion, the findings of the study on isthmus involution in moulting birds suggest that autophagy is a process confined to the early stages of degeneration, while apoptosis and/or necrosis occur in the terminal stages of regression.

1. Introduction

Egg production in commercial layers commences at 18 weeks of age, peaks when the birds are 32 weeks old, and then decreases as the birds begin moulting at 65 to 75 weeks of age (Hy-line International, 2016). Moulting is typically defined as the seasonal loss and replacement of body feathers, a process accompanied by the partial or complete regression of the reproductive tract (Berry, 2003). Commercially several methods, such as feed withdrawal, water deprivation and feeding low caloric or high salt diets are used to induce or force moulting, resulting in the complete cessation of egg production for a period of 6 to 8 weeks (Webster, 2003; McCowan *et al*., 2006). The re-introduction of a balanced feed and an increase in photoperiod leads to the recrudescence of the reproductive tract and a high level of egg production (Bell, 2003).

Although commercially profitable, induced moulting is generally not considered humane (Ruszler, 1998; Bell, 2003; Koelkebeck and Anderson, 2007). In addition, induced moulting, using feed withdrawal methods, has been associated with the production of *Salmonella enteritidis* contaminated eggs (Holt, 2003; Ricke, 2003). In contrast to induced moulting, commercial layers naturally undergo a moult at 65 to 75 weeks of age (Hy-Line International, 2016). Although natural moulting does not result in high egg production post-moult, this process avoids animal welfare and food safety issues. For these reasons it would be preferable for the poultry industry to use natural instead of induced moulting methods. However, in order for hens to achieve maximum egg production following natural moulting it is necessary to manipulate this process, for example using varying photoperiods. Such manipulation requires an understanding of the cellular changes, at light microscopic and ultrastructural levels, occurring during natural moulting.

It is known that oviductal involution during moulting occurs via apoptosis and necrosis (Heryanto *et al*., 1997; Sundaresan *et al*., 2008). The term “apoptosis” was introduced by Kerr and co-workers (1972) to describe a mode of cell death characterized by specific ultrastructural features, which include: cytoplasmic condensation; nuclear chromatin aggregation and margination; nuclear fragmentation and the formation of apoptotic bodies. Apoptotic bodies are composed of cytoplasmic fragments, with or without nuclear fragments (Kerr *et al.,* 1972; Sandow *et al*., 1979; Verhage *et al*., 1984). Apoptosis is activated by multiple and varied triggers, ranging from toxins (Kerr *et al*., 1972; Kerr, 2002; Lockshin and Zakeri, 2004; Elmore, 2007), to hormonal manipulations (Sandow *et al*., 1979; Thompson, 1994), and changes in hormone levels during the oestrous cycle (Verhage *et al*., 1984; Sato *et al*., 1997; Wick and Kress, 2002), menstrual cycle (Verma, 1983) and pregnancy (Parr *et al*., 1987;

Demir *et al*., 2002). Researchers have proposed that the processes of apoptosis and necrosis are related (Zeiss, 2003). In this regard it has been shown that cells in which apoptosis has been initiated may instead undergo programmed necrosis in the event of an inadequate supply of caspases (Denecker *et al*., 2001) and/or adenosine triphosphate (Leist *et al*., 1997).

Extensive research has been conducted on the physiological basis of moulting and the various hormonal, as well as immunological interactions involved in the process (Sundaresan *et al*., 2007ab; Berry, 2003; Webster, 2003). In addition, the reduction in the number of oviductal cells, as well as the loss in reproductive tract weight associated with moulting has been documented (Yu and Marquardt, 1974). However, the ultrastructural alterations in the various regions of the oviduct associated with the hormonal changes and oviductal weight loss have not been investigated.

The isthmus region of the oviduct is responsible for the secretion of a shell membrane, composed of inner and outer layers, around the developing egg (Hoffer, 1971; Draper *et al*., 1972). In comparison with the other regions of the oviduct relatively few ultrastructural studies have been conducted on the isthmus. Furthermore, there is currently no published information on ultrastructural changes in the isthmus in moulting birds. The purpose of the current study was to provide a detailed description of the isthmus in laying hens, and to further describe changes associated with natural moulting.

2. Materials and Methods

2.1. Animals and management

A total of 10 laying (32 weeks old) and 10 moulting (75 weeks old) commercial hens (Hy-line W-36) were used in the present study. The birds were maintained in pens under a light regime of 16 h light: 8 h dark. Feed and water were provided *ad libitum*. The birds were killed by decapitation. All the procedures used in this study were approved by the Animal Ethics Committee of the University of Pretoria (approval number AEC V002/17).

2.2. Necropsy

The thoraco-abdominal cavity was cut open, and the oviduct was removed. The oviduct was cut longitudinally to expose the luminal surface of the organ and the position, as well as the developmental stage of the forming egg in the oviduct was noted. Developing eggs were present in the oviducts of all laying birds. Eggs were situated in the isthmus regions of the oviducts in three of the ten laying birds. No eggs were present in the oviducts of the moulting birds. Tissue samples were collected from the middle region of the isthmus.

2.3. Light microscopy

Tissue samples were immersion-fixed in 10% buffered neutral formalin for 5 days. After fixation, the tissues were processed routinely for histology and embedded in paraffin wax. Sections (5μm thick) were stained with haematoxylin and eosin. The stained sections were viewed and photographed using an Olympus BX-63 microscope.

2.4. Terminal deoxynucleotidyl transferase (TdT)- mediated deoxyuridine 5'-triphosphate (dUTP) nick-end labelling (TUNEL)

Apoptotic cells were detected using the TUNEL assay. The technique was performed on 5 μm thick sections using an ApopTag plus peroxidase *in situ* apoptosis detection kit (Millipore, Temecula, USA) following the manufacturer's instructions. Sections were deparaffinized, rehydrated through a graded series of ethanol and washed in 0.01M phosphate buffered saline solution (PBS, pH 7.4). The sections were then pretreated with proteinase K (20μg/ml) for 15 min at room temperature. The slides were then rinsed in two changes of distilled water for 2 min each. Endogenous peroxidase activity was blocked using a 3% (v/v) hydrogen peroxidase solution in PBS for 5 min. The slides were then rinsed in PBS for 5 min. Thereafter, the sections were incubated with an equilibration buffer (ApopTag plus peroxidase kit, Millipore, Temecula, USA) at room temperature for 10 min. Excess buffer was tapped off the sections before the application of working strength TdT enzyme (77μl reaction buffer and 33μl TdT enzyme). The sections were then incubated in a humidified chamber at 37^0C for 1h. The reaction was stopped by rinsing the sections in stop/wash buffer (ApopTag plus peroxidase kit, Millipore, Temecula, USA) for 10 min at room temperature. The sections were then rinsed in three changes of PBS for 1 min each. The excess buffer was tapped off before the application of anti-digoxignenin conjugate (ApopTag plus peroxidase kit, Millipore, Temecula, USA). The sections were then incubated in a humidified chamber for 30 min at room temperature. The sections were then rinsed in four changes of PBS for 2 min each. Excess buffer was then tapped off the slides. Apoptotic nuclei were visualized after the addition of a diaminobenzidine solution (ApopTag plus peroxidase kit, Millipore, Temecula, USA). The sections were then rinsed in distilled water and counterstained with 0.5% (w:v) methyl green for 10

min at room temperature. The slides were then rinsed in distilled water and then dehydrated in 100% N-butanol and cleared in xylene.

In the negative controls TdT enzyme reagent was replaced with distilled water. Rat mammary gland (supplied with the ApopTag plus peroxidase kit) was used as a positive control.

The number of TUNEL positive nuclei in 100μm lengths of epithelium in 15 random microscopic fields per bird were counted using an image analyser system (CellSens dimension software) connected to an Olympus BX-63 microscope.

2.5. Statistical analysis

TUNEL positive nuclei counts in laying and moulting hens were analyzed with the Independent samples t-test using IBM SPSS 25 software. A P value of <0.05 was considered to be statistically significant.

2.6. Scanning electron microscopy (SEM)

The SEM procedure was carried out as previously described (Madekurozwa, 2007). In brief, tissue samples for SEM were pinned flat, rinsed in 0.075M phosphate buffer (pH 7.4) and then fixed in buffered 2.5% glutaraldehyde. After fixation the samples were rinsed in phosphate buffer, and then post-fixed in 0.5% aqueous osmium tetroxide. The samples were then dehydrated in a graded series of ethanol before being dried in a critical point chamber with liquid CO_2. The samples were then coated with carbon and viewed using a Zeiss Crossbeam 540 FEG scanning electron microscope operated at 3kv.

2.7. Transmission electron microscopy (TEM)

The TEM procedure was conducted as described in a previous study (Madekurozwa, 2007). Tissue samples were immersion-fixed in 2.5% glutaraldehyde in 0.075M phosphate buffer (pH 7.4) for 24 hours. Thereafter, the tissue samples were post-fixed in 0.5% osmium tetroxide for 2 hours. The tissue samples were then rinsed in phosphate buffer (pH 7.4), dehydrated in a series of ethanol concentrations and embedded in epoxy:resin at a ratio of 1:2 for 1 hour, 1:1 for 2 hours and 100% resin overnight. Semi-thin sections were cut using a glass knife and stained with toluidine blue. Ultra-thin sections were cut using a diamond knife, stained with lead acetate and counter stained with uranyl citrate. The samples were viewed with a Philips CM10 transmission electron microscope (FEI, The Netherlands), fitted with an Olympus Mega View III imaging system.

3. Results

3.1. Light microscopy

3.1.1. Haematoxylin and eosin-stained sections

The mucosa of the isthmus was formed by leaf-shaped primary folds with regularly-placed secondary folds. Lining the mucosal folds in laying birds was a pseudostratified columnar epithelium, composed of ciliated and non-ciliated cells (Fig. 1a). The luminal epithelium in moulting birds consisted of vacuolated cells, intermingled with cells displaying an apparently normal morphology (Fig. 1b). Loss of cilia was evident at the light microscopic level in haematoxylin and eosin-stained sections.

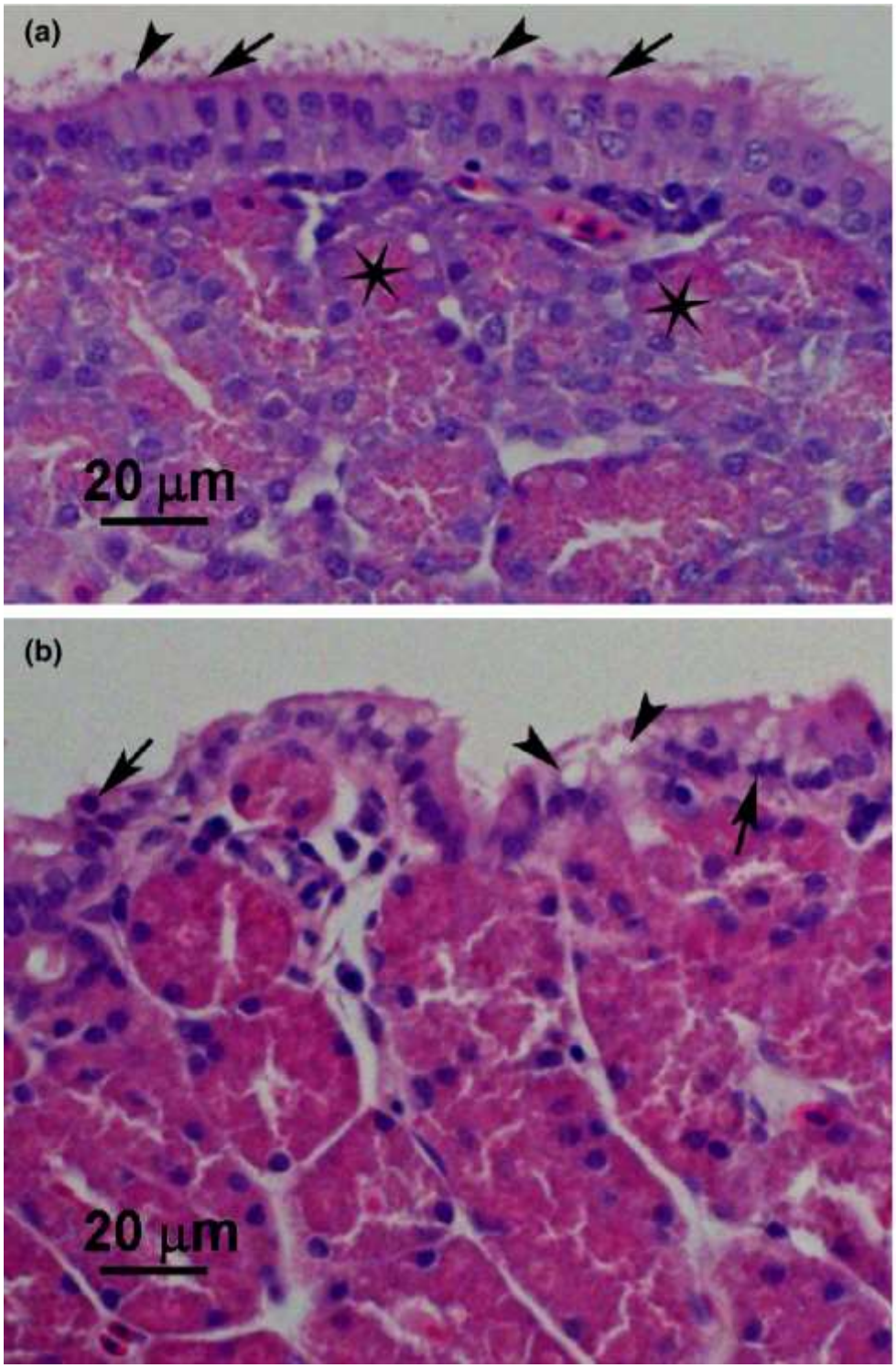

Fig. 1. Light photomicrographs of the isthmus in laying (A) and moulting (B) birds. Haematoxylin and eosin.

A. Ciliated (arrows) and non-ciliated (arrowheads) luminal epithelial cells in a laying bird. Asterisks: tubular glands.

B. Pyknotic nuclei (arrows) and vacuolated cytoplasm (arrowheads) in the degenerating luminal epithelium of a moulting bird.

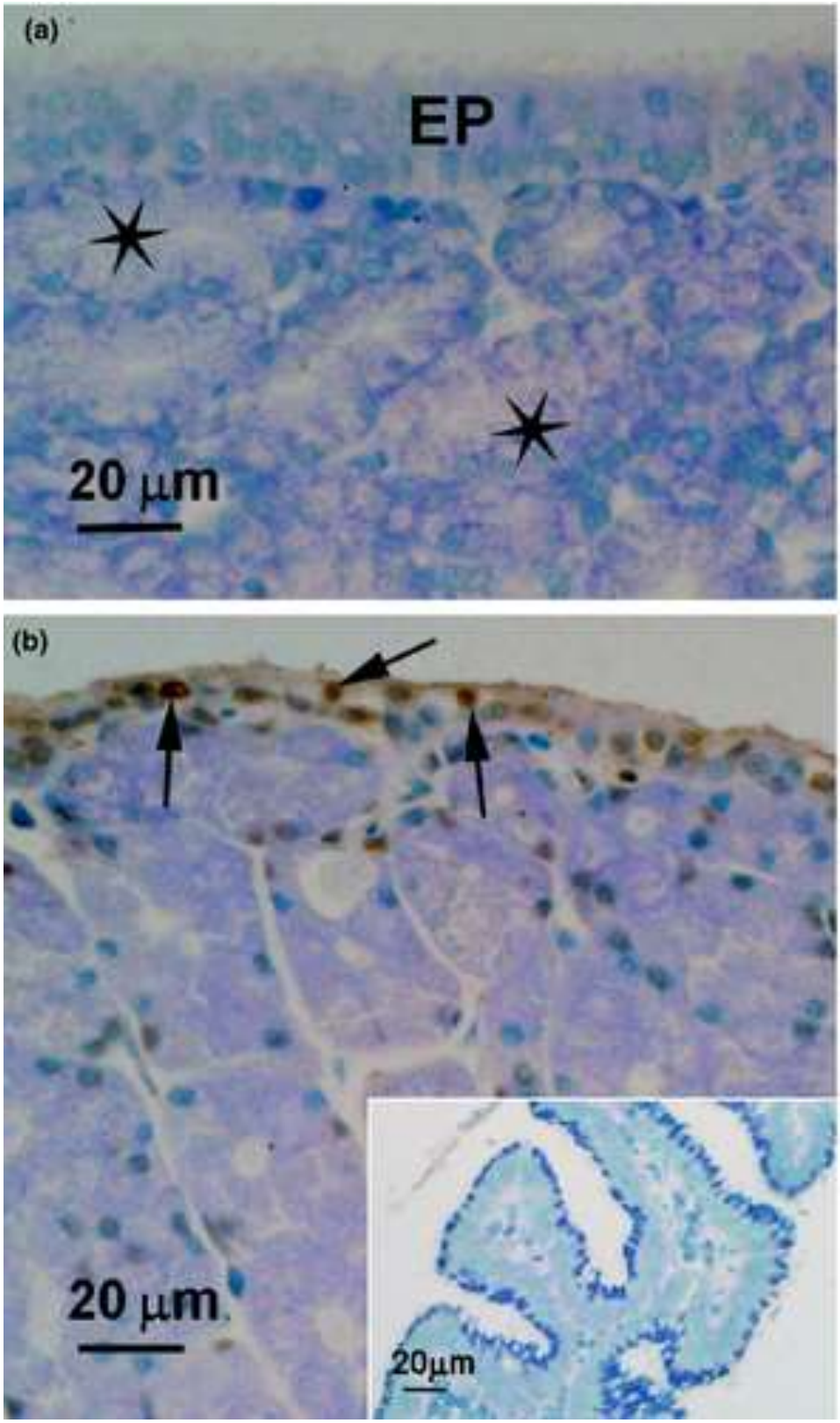

Fig. 2. TUNEL-stained sections of the isthmus in laying (A) and moulting (B) birds.

A. TUNEL-negative luminal epithelium (EP) in a laying bird. Asterisks: tubular glands.

B. TUNEL-positive nuclei (arrows) in the luminal epithelium of a moulting bird. Inset: Negative control.

3.1.2. TUNEL-stained sections

TUNEL-staining in the luminal epithelia of laying birds was negligible (Fig. 2a). In moulting birds, nuclei exhibiting varying intensities of TUNEL-staining occurred throughout the luminal epithelia during the intermediate and advanced stages of regression (Fig. 2b).

The mean +/- standard deviation of TUNEL-positive cells in laying birds was 0.6 +/- 0.84 (N = 10), and 11.70 +/- 2.75 (N = 10) for moulting birds. The standard error mean was 0.26 for laying birds and 0.86 for the moulting birds. The results of the Independent samples t-test revealed a significant difference between the mean counts of TUNEL-positive cells in laying and moulting hens. The mean difference in TUNEL cell counts was 11.1, while the standard error difference was 0.90 at a 95% confidence interval. In summary the number of TUNEL-stained nuclei in the luminal epithelia of moulting birds was significantly higher than in laying birds.

3.2. Scanning electron microscopy

3.2.1. Laying birds

The luminal surface in laying birds was lined by ciliated and non-ciliated cells, with the former being more predominant. The ciliated cells displayed uniform cilia, which partially obscured adjacent non-ciliated cells (Fig. 3a). In addition, microvilli were visible among the cilia lining ciliated cells. Non-ciliated cells were covered by a dense mat of microvilli, except in areas where apical protrusions occurred (Fig. 3b).

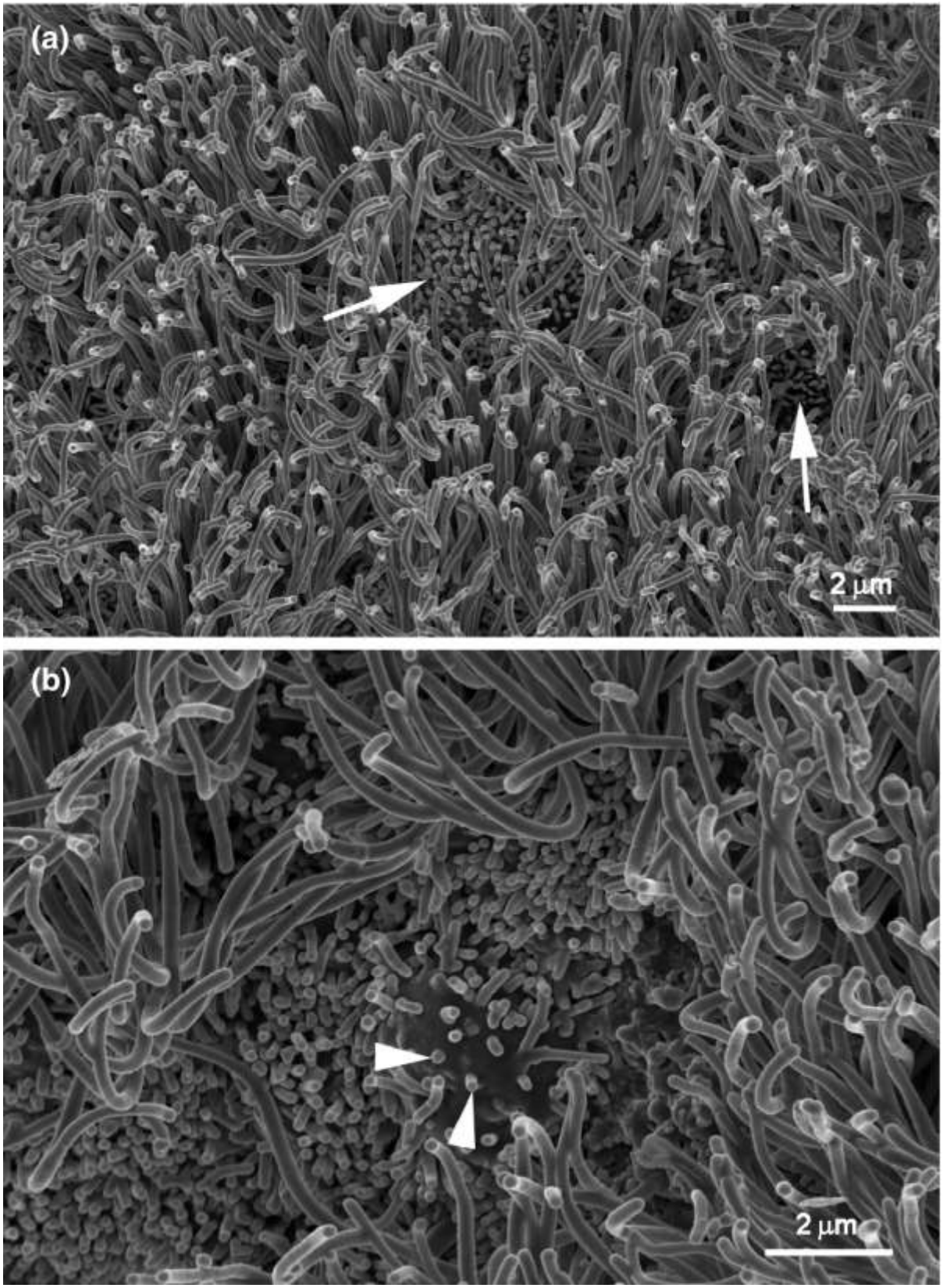

Fig. 3. Scanning electron photomicrographs of the isthmus surface in laying birds.

A. Cilia partially obscure non-ciliated cells (arrows).

B. A protrusion from a non-ciliated cell exhibits a few microvilli (arrowheads).

3.2.2. Moulting birds

Morphological changes involving ciliated cells included swollen cilia (Fig. 4a), cilia packets (Fig. 4b), as well as the presence of a low number of cilia (Fig. 4c). Cilia packets were composed of an aggregation of cilia partially enclosed in a membrane. Cilia appeared to be progressively incorporated into the distal regions of the cilia packets (Fig. 4b).

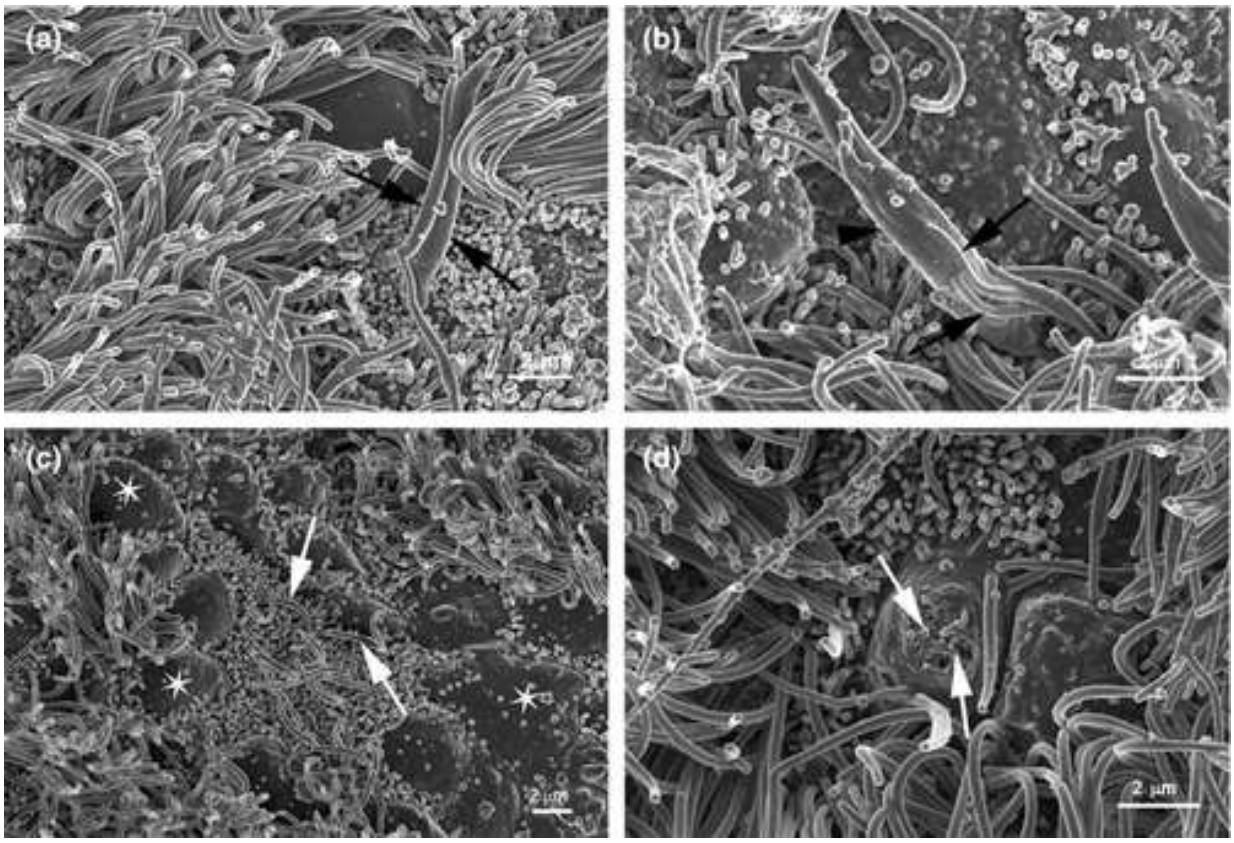

Fig. 4. Scanning electron photomicrographs of the isthmus surface in moulting birds. **A**. Arrows: swollen cilia.

B. Cilia (arrows) merging with a cilia packet (arrowhead).

C. Ciliated cell displaying a few cilia (arrows). Asterisks: non-ciliated cells.

D. Cytoplasmic contents (arrows) of a non-ciliated cell are exposed due to the absence of an apical plasma membrane.

A few short microvilli extended from the apical protrusions of non-ciliated cells in moulting birds. In addition, SEM revealed the presence of non-ciliated cells which lacked apical plasma membranes (Fig. 4d). Cytoplasmic organelles were visible due to the absence of the apical plasma membranes (Fig. 4d).

3.3. Transmission electron microscopy

3.3.1. Ciliated cells

Ciliated cells were typically “goblet” shaped with a broad apical region, which contained the nucleus, and a narrow basal area in contact with the basal lamina (Fig. 5). The apical cytoplasm contained numerous elongated mitochondria, which were situated close to ciliary rootlets (Fig. 6a). Contained within the supranuclear cytoplasm were a few rough endoplasmic reticulum (RER) cisternae (Fig. 6a), ribosomes, accumulations of vesicles, electron dense bodies and bundles of fibrils. The narrow basal regions of the ciliated cells contained mitochondria, RER cisternae and ribosomes (Fig. 6b).

The foremost ultrastructural alterations in ciliated cells during the early stages of degeneration were the presence of supranuclear autophagosomes, multivesicular bodies and autolysosomes. Degenerating ciliated cells, with either electron lucent or electron dense cytoplasm, were observed during the intermediate and late stages of regression. During the intermediate stages of regression, the electron lucent cells contained irregular-shaped euchromatic nuclei, disintegrating mitochondria, and numerous vacuoles. These features of involution were concomitant with a loss of cilia, which occurred through the budding and release of cilia packets into the lumen of the isthmus (Fig. 7a). The cilia packets contained ciliary shafts, while basal bodies and rootlets were retained in the apical cytoplasm of the ciliated cells. In the advanced

stages of regression, the apical plasma membranes of the electron lucent ciliated cells disintegrated, which resulted in the release of cellular contents into the lumen of the isthmus.

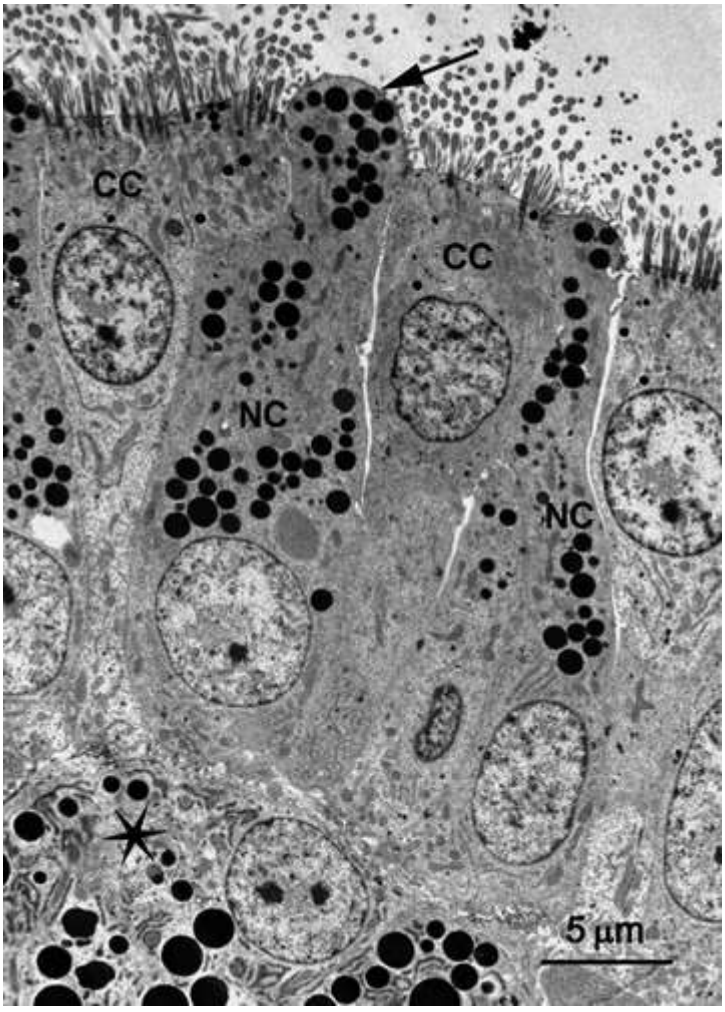

Fig. 5. Transmission electron photomicrograph of luminal epithelial cells in the isthmus of a laying hen. CC: ciliated cells. NC: non-ciliated cells. Arrow: apical protrusion. Asterisk: tubular gland.

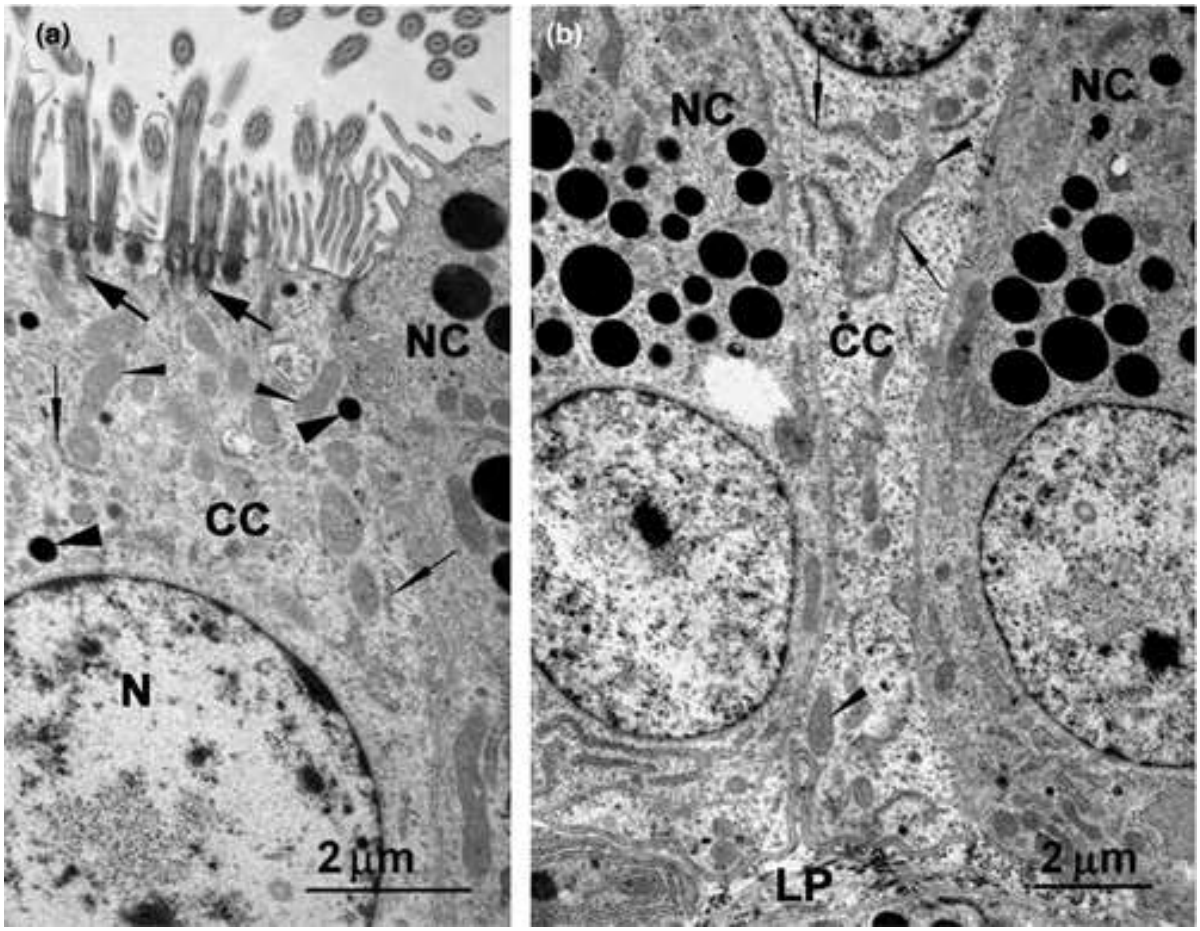

Fig. 6. Transmission electron photomicrographs of the apical (A) and basal (b) regions of luminal epithelial cells in the isthmus of a laying hen. NC: non-ciliated cells. CC: ciliated cells. Thick arrows: cilia rootlets. Thin arrows: RER profiles. Thick arrowheads: electron dense bodies. Thin arrowheads: mitochondria. N: nucleus. LP: lamina propria.

Electron dense degenerating ciliated cells in the isthmus of moulting birds contained condensed cytoplasm, disintegrating mitochondria, enlarged Golgi complex saccules, dilated RER cisternae, and irregular-shaped nuclei with chromatin aggregation and margination (Fig. 7b). Although the apical plasma membranes of these cells displayed remnants of cilia, there was no evidence of cilia packet formation.

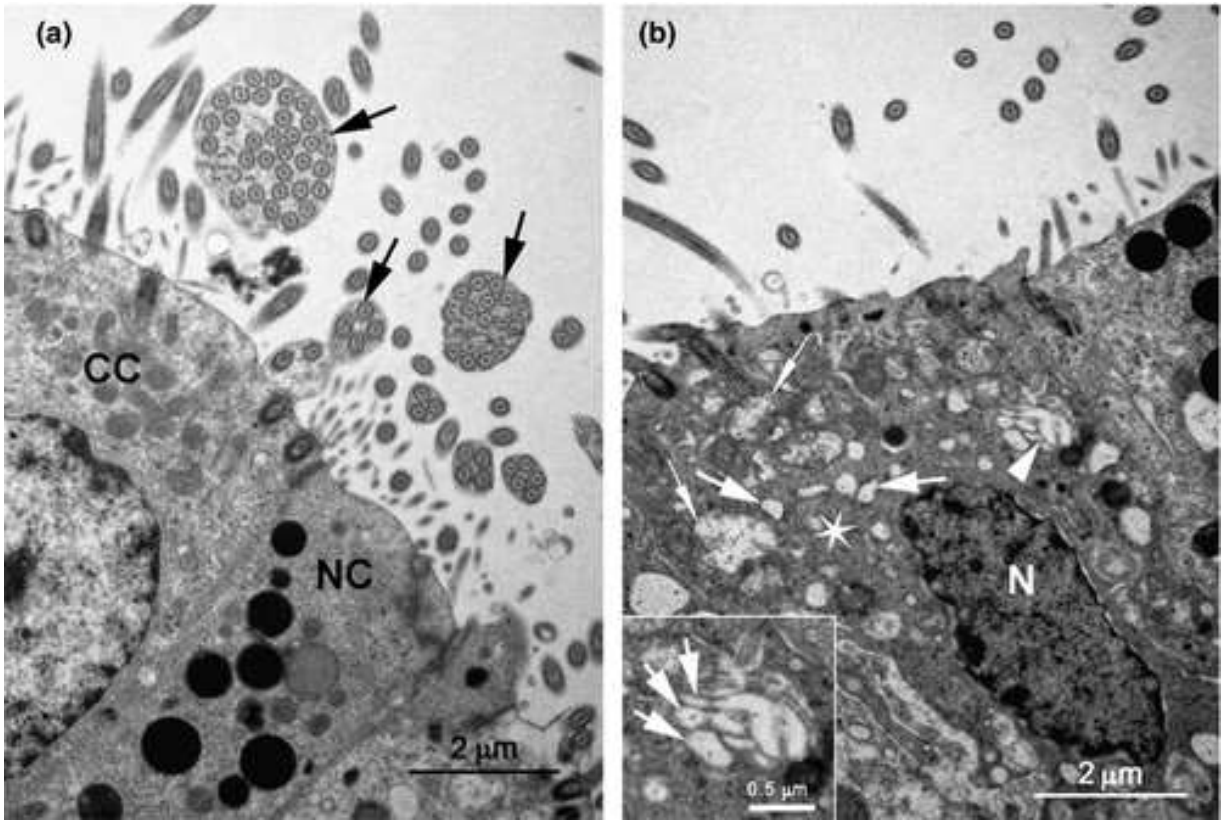

Fig. 7. Transmission electron photomicrographs of luminal epithelial cells in the isthmus of moulting hens.

A. CC: ciliated cell. NC: non-ciliated cell. Arrows: cilia packets.

B: Asterisk: condensed cytoplasm. Thick arrows: dilated RER cisternae. Thin arrows: disintegrating mitochondria. Arrowhead: dilated Golgi complex tubules. N: condensed nucleus. Inset: Arrows: Stacked dilated saccules of a Golgi complex.

3.3.2. *Non-ciliated cells*

Non-ciliated cells contained euchromatic nuclei with one or two prominent nucleoli (Fig. 5). Distributed throughout the cytoplasm were mitochondria and RER cisternae. Masses of secretory granules were located in the immediate supranuclear area, as well as in apical cytoplasm. Multiple Golgi complexes occupied the intervening cytoplasm between the two accumulations of secretory granules (Fig. 8a). Apical cytoplasmic protrusions containing secretory granules were present when a

developing egg was located in the infundibulum and magnum regions of the oviduct (Fig. 5). In contrast, relatively few apical protrusions were observed when a developing egg was in the isthmus or shell gland.

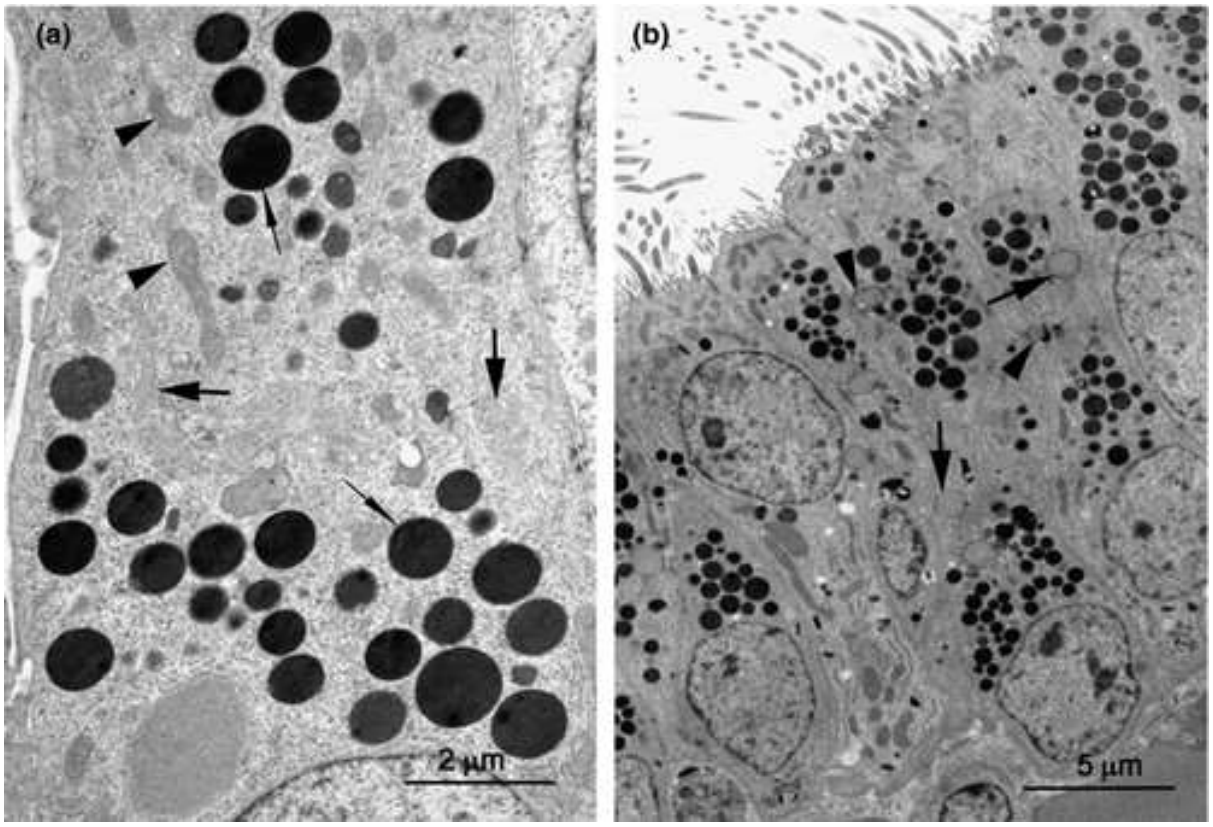

Fig. 8. Transmission electron photomicrographs of the luminal epithelium in the isthmus of laying (A) and moulting (B) birds.

A. Golgi complexes (thick arrows), secretory granules (thin arrows) and mitochondria (arrowheads) in a non-ciliated cell.

B. Autophagosomes (arrows) and autolysosomes (arrowheads) in non-ciliated cells.

The cytoplasm in non-ciliated cells undergoing early degeneration contained autophagosomes, lysosomes, autolysosomes and small Golgi complexes (Fig. 8b). The autophagosomes contained lipid droplets of intermediate electron density. Although most secretory granules displayed an apparently normal appearance, occasional degenerating granules were observed. These irregular-shaped, regressing secretory granules contained clumps of electron dense material.

During the intermediate stages of regression, the luminal surfaces of non-ciliated cells were thrown into broad cytoplasmic processes (Fig. 9a). A few secretory granules of various sizes, as well as several dilated profiles of RER were located directly below the apical plasma membrane (Fig. 9a).

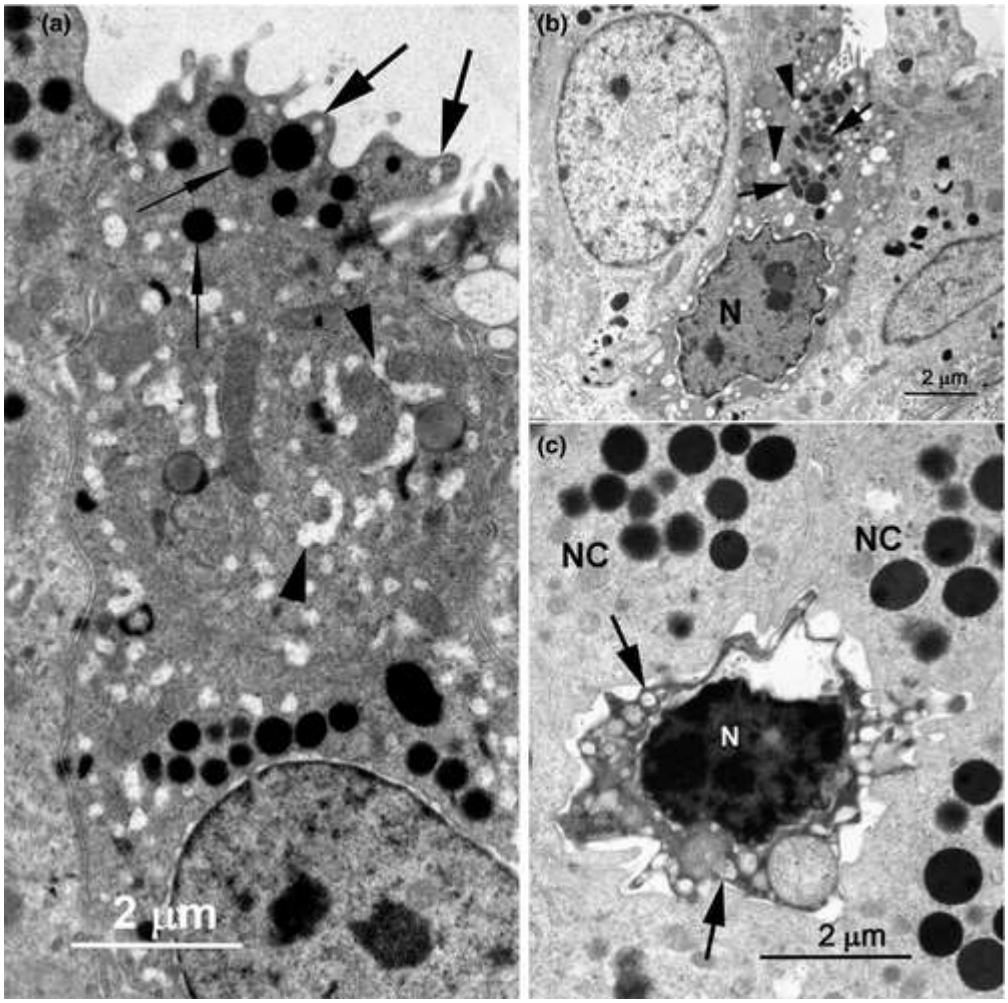

Fig. 9. Transmission electron photomicrographs of the luminal epithelium in the isthmus of moulting birds.

A. Thick arrows: cytoplasmic protrusions. Thin arrows: secretory granules. Arrowheads: dilated RER cisternae.

B. Arrows: pleomorphic secretory granules. Arrowheads: vacuoles. N: condensed, irregular-shaped nucleus.

C. Arrows: apoptotic body. N: Nuclear fragment. NC: non-ciliated cells.

In the later stages of degeneration non-ciliated cells were characterized by the presence of condensed cytoplasm with numerous vacuoles and pleomorphic secretory granules (Fig. 9b). In addition, these degenerating cells contained irregular-shaped condensed nuclei (Fig. 9b).

3.3.3. Apoptotic bodies

Although ciliated and non-ciliated cells were easily distinguishable in the early, intermediate, and advanced stages of degeneration, it was not possible to determine with certainty which of these two cell types gave rise to apoptotic bodies. The apoptotic bodies were characterized by the presence of irregular-shaped, electron dense nucleic fragments enclosed in condensed cytoplasm (Fig. 9c).

3.3.4. Mitochondrial cells

Two forms of mitochondrial cells (types 1 and 2) were observed in the luminal epithelium of the isthmus. Type 1 mitochondrial cells were tall columnar with basally-located nuclei. With the exception of the apical regions, the entire supranuclear cytoplasm of type 1 mitochondrial cells was filled with elongated mitochondria. In addition, these cells displayed apical protrusions which were associated with occasional microvilli (Fig. 10a). The area of cytoplasm below the apical protrusions contained a meshwork of fibrils with numerous vesicles (Fig. 10a).

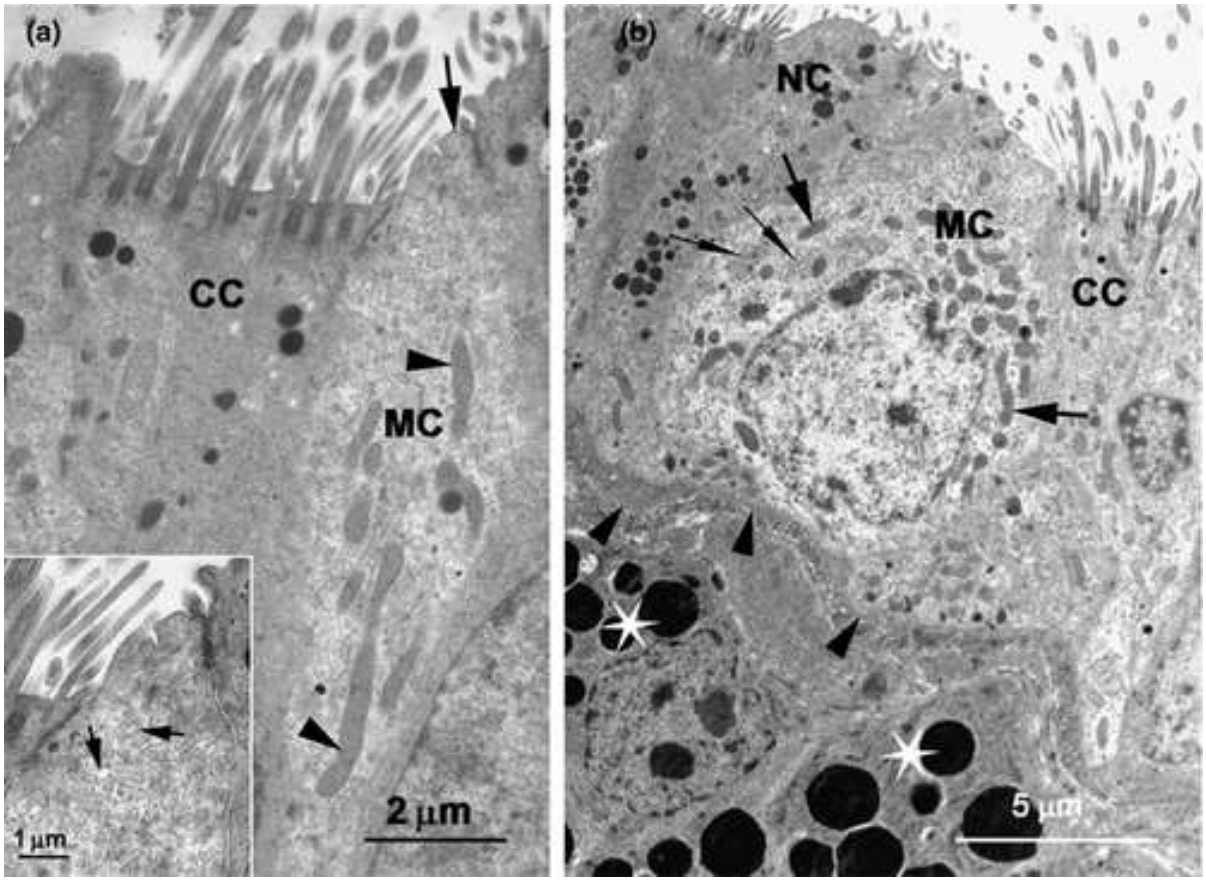

Fig. 10. Transmission electron photomicrographs of type 1 (A) and type 2 (B) mitochondrial (MC) cells in the isthmus of laying birds. Ciliated (CC) and non-ciliated (NC) cells border the mitochondrial cells.

A. Arrow: Apical protrusion. Arrowheads: mitochondria. Inset: Several vacuoles (arrows) are present in the apical region of the mitochondrial cell.

B. Thick arrows: mitochondria. Thin arrows: Golgi complexes. Arrowheads: basal lamina. Asterisks: tubular gland cells.

Type 2 mitochondrial cells were pyramidal in shape, with narrow apical and broad basal regions (Fig. 10b). These cells contained centrally-located indented nuclei. Most of the mitochondria in these cells were located in the immediate supranuclear region, while the apical cytoplasm was devoid of mitochondria. Clear vesicles were distributed throughout the cytoplasm, but were particularly numerous in the apical cytoplasm, which did not protrude into the lumen. In addition, a few coated vesicles were observed among the clear vesicles. Numerous short smooth

endoplasmic reticulum (SER) profiles were observed in the cytoplasm directly below the vesicles. Multiple Golgi complexes, numerous ribosomes and a few RER cisternae were observed in the perinuclear cytoplasm (Fig. 10b). Type 1 and 2 mitochondrial cells appeared to undergo similar processes of degeneration. As in laying birds, the cytoplasm in the mitochondrial cells of moulting hens was dominated by elongated mitochondria with well-defined cristae (Fig. 11a). Mitochondrial cells in the initial stages of regression variably displayed multivesicular bodies, autophagosomes, autolysosomes, vacuoles and residual bodies (Figs. 11a & b). The autophagosomes contained lipid droplets, which were partially enclosed by degenerating mitochondria (Fig. 11b).

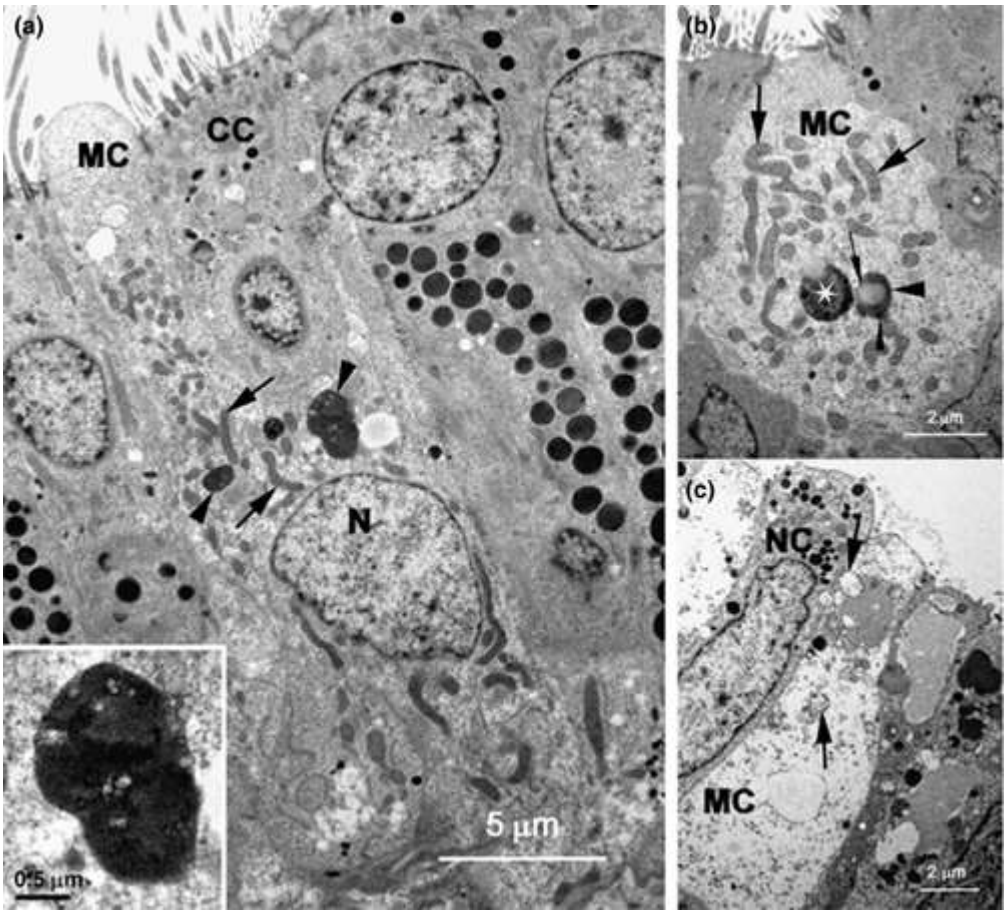

Fig. 11. Transmission electron photomicrographs of type 1 (A & C) and type 2 (B) mitochondrial (MC) cells in the isthmus of moulting birds. Degenerating ciliated (CC) and non-ciliated (NC) cells border the mitochondrial cells.

A. Arrows: mitochondria. Arrowheads: residual bodies. N: nucleus. Inset: Higher magnification of a residual body.

B. Thick arrows: mitochondria. Asterisk: autolysosome. Note the second autolysosome composed of a lipid droplet (thin arrow), degenerating mitochondrion (thick arrowhead) and a lysosome (thin arrowhead).

C. Arrows: mitochondria.

Numerous swollen, disintegrating mitochondria and dilated RER cisternae were present in the intermediate stages of regression. The nuclei in these regressing cells displayed an apparently normal appearance.

Mitochondrial cells in the advanced stages of regression contained electron lucent cytoplasm, which enclosed a few swollen mitochondria (Fig. 11c). Apical plasma membranes were still intact in these late stages of degeneration.

4. Discussion

4.1. Ciliated cells

The histology and ultrastructure of ciliated cells in the isthmus of laying birds in the current study was in agreement with previous studies on the domestic fowl (Draper *et al*., 1972; Solomon, 1975; Chousalkar and Roberts, 2008).

Signs of autophagy were observed in the early stages of regression in the ciliated cells of moulting birds, while characteristic features of apoptosis and necrosis were evident during the intermediate, as well as advanced degenerative phases. The occurrence of apoptotic cells during the intermediate and advanced stages of regression was confirmed using the TUNEL assay. In the present study autophagy was marked by the occurrence of autophagosomes, multivesicular bodies, and autolysosomes. Similar autophagy- associated inclusions have been reported in the

endometrial luminal epithelium of the rat undergoing estrus (Spornitz *et al*.,1994) and in the opossum during metestrus (Wick and Kress, 2002). According to Eskelinen (2008) multivesicular bodies are an integral component of autophagy, with autophagosomes fusing with these structures in order to obtain regulatory proteins, such as lysosomal membrane proteins, which are involved in the process of autophagosomal-lysosomal fusion.

An interesting finding of the present study was the observation of ciliated cells undergoing cell death via either apoptosis or necrosis. The characteristic features of apoptosis exhibited by these degenerating ciliated cells included: cytoplasmic condensation, as well as nuclear chromatin aggregation and margination. Apoptosis, culminating in the formation of apoptotic bodies, is also the most common form of cell death encountered in the luminal endometrial epithelia of mammalian species during the oestrous and menstrual cycles (Sandow *et al*., 1979; Verhage *et al*., 1984; Spornitz *et al*., 1994; Sato *et al*., 1997; Demir *et al.*, 2002). In addition, studies have shown that apoptosis is involved in oviductal regression of moulting hens (Heryanto *et al*., 1997; Jeong *et al*., 2013).

In addition to apoptosis, there is evidence that involution of the oviduct during induced moulting also occurs via necrosis (Heryanto *et al*., 1997). Using acid phosphatase as a marker for necrosis, Heryanto and co-workers (1997) demonstrated a moderate staining for this enzyme in the isthmus of domestic fowls 2 and 7 days post-induction of moulting. These authors concluded that apoptosis occurs during the earlier stages of oviductal regression, whereas necrosis is involved in the later degenerative phases. However, the use of TEM in the current study showed that apoptosis and necrosis occur concurrently in degenerating ciliated cells of the isthmus during natural moulting.

The loss of cilia via the formation and extrusion of cilia packets was a further notable feature of necrotic ciliated cells in the present study. The TEM study showed the budding and release of cilia packets, while the SEM investigation revealed the lateral incorporation of cilia into these structures. Deciliation via cilia packet formation has previously been reported in the rat (Reeder and Shirley, 1999) and opossum (Wick and Kress, 2002). These studies proposed that changes in the rate of cilia packet formation may be related to fluctuations in the circulating levels of ovarian or reproductive hormones (Reeder and Shirley, 1999; Wick and Kress, 2002).

4.2. Non-ciliated cells

Ultrastructurally, the appearance of non-ciliated cells in the current study did not differ from previous descriptions of similar cells in the domestic fowl (Draper *et al*.,1972; Solomon, 1975; Chousalkar and Roberts, 2008) and Japanese quail (Hoffer, 1971). As noted by Draper and co-workers (1972), as well as Chousalkar and Roberts (2008), the ultrastructure of non-ciliated cells in the isthmus varies according to the position of a developing egg in the oviduct. In agreement with the findings of Chousalkar and Roberts (2008), apical protrusions of the non-ciliated cells, in the current study, were prominent prior to the arrival of an egg in the isthmus. Thereafter, few apical protrusions were observed even though the cells were not completely devoid of secretory granules.

The early stages of degeneration in non-ciliated cells were characterized by the presence of multiple lipid droplet-autophagosomes. Classic features of apoptosis, which included nuclear and cytoplasmic condensation, were observed in the later stages of regression. The occurrence of lipid droplets in apoptotic cells has been well-documented in mammalian species (Finstad *et al*., 1998, 2000; Hakumaki and

Kauppinen, 2000). In addition, studies have shown that in mammals these lipid droplets, which contain triglycerides and cholesteryl esters, develop from endoplasmic reticulum (Hakumaki and Kauppinen, 2000; Walther and Farese, 2009). Further investigations are required to determine the origin of lipid droplets in degenerating cells in the avian oviduct. Furthermore, the biochemical content of these lipid droplets in the domestic fowl needs to elucidated.

4.3. Mitochondrial cells

Mitochondrial cells in the isthmus of the domestic fowl were first described by Draper and co-workers (1972). Solomon (1975) later confirmed the occurrence of these cells, although a description was not provided. The columnar-shaped mitochondrial cells described in the study by Draper *et al*. (1972) contained numerous mitochondria and apical protrusions with vesicles. Based on this description, the cells observed by Draper and co-researchers (1972) correspond to the mitochondrial cells designated as type 1 in the current study. In addition to these type 1 cells, the present study described a second form of mitochondrial cell (type 2), which has not been previously described. In contrast to type 1 cells, type 2 mitochondrial cells did not display apical protrusions, despite the luminal cytoplasm containing numerous vesicles. In addition, type 2 cells contained SER cisternae in the sub-apical cytoplasm, an area dominated by fibrils in type 1 cells. Furthermore, type 2 cells contained multiple Golgi complexes, a feature which was not observed in type 1 cells.

Even though mitochondrial cells, in the isthmus of the domestic fowl, were first described over four decades ago, relatively little is known about the function of these cells. Draper and co-workers (1972) suggested that based on morphological features

mitochondrial cells may have a role in ion secretion. The numerous vesicles and mitochondria observed in the present study support this notion.

In moulting birds features of autophagy, such as autophagosomes, multivesicular bodies and autolysosomes, were observed in the early stages of degeneration of mitochondrial cells. As in non-ciliated cells the central regions of the autophagosomes were formed by lipid droplets. An interesting feature of mitochondrial cells was the subsequent incorporation of degenerating mitochondria into the autophagosomes.

In contrast to ciliated and non-ciliated cells, mitochondrial cells in the current study did not appear to display any features of apoptosis. Instead cell death in mitochondrial cells occurred via necrosis, which was characterised by the presence of swollen mitochondria, dilated RER cisternae and cytolysis.

5. Conclusion

Observations made in the current study indicate that cells of the luminal epithelium of the isthmus regress via autophagy, apoptosis and necrosis. Autophagy occurred during the earlier degenerative stages, while apoptosis and necrosis were evident in the more advanced regressive phases. Furthermore, although apoptosis occurred in non-ciliated cells and necrosis in mitochondrial cells, both modes of cell death were identified in ciliated cells. The factors or stimuli that determine whether a cell undergoes death via apoptosis or necrosis need to be elucidated.

Acknowledgements

The authors thank University of Pretoria technical staff: Lizette du Plessis, Antoinette Buys (Electron Microscope unit, Onderstepoort campus) and Erna van Wilpe

(Laboratory for Microscopy and Microanalysis, Hatfield campus), for their assistance and expertise. The University of Pretoria is gratefully acknowledged for the MSc postgraduate bursary awarded to Mike Mpango. This study was funded by the National Research Foundation of South Africa (grant #N00188).

References

Bell, D.D., 2003: Historical and current molting practices in the U.S. table egg industry. Poult. Sci. **82,** 965-970.

Berry, W.D., 2003: The physiology of induced molting. Poult. Sci. **82,** 971-980.

Chousalkar, K.K., J.R. Roberts, 2008: Ultrastructural changes in the oviduct of the laying hen during the laying cycle. Cell Tiss. Res. **332,** 349-358.

Demir, R., U.A. Kayisli, C. Celik-Ozenci, E.T. Korgun, A.Y. Demir-Weusten, A. Arici, 2002: Structural differentiation of human uterine luminal and glandular epithelium during early pregnancy: an ultrastructural and immunohistochemical study. Placenta. **23,** 672-684.

Denecker, G., D. Vecammen, M. Steemans, T. Vanden Berghe, G. Brouckaert, G. Van Loo, B. Zhivotovsky, W. Fiers, J. Grooten, W. Declercq, P. Vandenabeele, 2001: Death-receptor induced apoptotic and necrotic cell death: differential role of caspases and mitochondria. Cell Death Diff. **8,** 829-840.

Draper, M.H., M.F. Davidson, G.M. Wyburn, H.S. Johnston, 1972: The fine structure of the fibrous membrane forming region of the isthmus of the oviduct of *Gallus domesticus*. Quart. J. Exp. Physiol. **57,** 297-309.

Elmore, S., 2007: Apoptosis: a review of programmed cell death. Toxicol. Path. **35,** 495-516.

Eskelinen, E.L., 2008: New insights into the mechanisms of macroautophagy in mammalian cells. Int. Rev. Cell. Mol. Biol. **266,** 208-247.

Finstad, H.S., C.A. Drevon, M.A. Kulseth, A.V. Synstad, E. Knudsen, 1998: Cell proliferation, apoptosis and accumulation of lipid droplets in U937-1 cells incubated with eicosapentaenoic acid. Biochem. J. **336,** 451-459.

Finstad, H.S., H. Dyrendal, M.C. Wik Myhrstad, H. Heimli, C.A. Drevon, 2000: Uptake and activation of eicosapentaenoic acid are related to accumulation of triacyglycerol in Ramos cells dying from apoptosis. J. Lipid Res. **41,** 554-563.

Hakumaki, J.M., R.A. Kauppinen, 2000: H NMR visible lipids in the life and death of cells. TIBS. **25,** 357-362.

Heryanto, B., Y. Yoshimura, T. Tamura, T. Okamoto, 1997: Involvement of apoptosis and lysosomal hydrolase activity in the oviductal regression during induced moulting in chickens: a cytochemical study for end labelling of fragmented DNA and acid phosphatise. Poult. Sci. **76,** 67-72.

Hoffer, A.P., 1971: The ultrastructure and cytochemistry of the shell membrane-secreting region of the Japanese quail oviduct. Am. J. Anat. **131,** 253-287.

Holt, P.S., 2003: Molting and *Salmonella enteric* serovar *enteritidis* infection: the problem and some solutions. Poult. Sci. **82,** 1008-1010.

Hy-line International, 2016: Management guide. W-36 commercial layers. pp. 21-24. www.hyline.com

Jeong, W., W. Lim, S.E. Ahn, C-H. Lim, J-Y. Lee, S-M. Bae, J. Kim, F.W. Bazer,

G. Song, 2013: Recrudescence mechanisms and gene expression profile of the reproductive tracts from chickens during the molting period. PLOS one. 8, e76784.

Kerr, J.F.R., 2002: History of the events leading to the formulation of the apoptosis concept. Toxicol. **181,** 471-474.

Kerr, J.F.R., A.H. Wyllie, A.R. Currie, 1972: Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. Br. J. Cancer. **26,** 239-257.

Koelkebeck, K.W., K.E. Anderson, 2007: Molting layers – alternative methods and their effectiveness. Poult. Sci. **86,** 1260-1264.

Leist, M., B. Single, A.F. Castoldi, S. Kuhnle, P. Nicotera, 1997: Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J. Exp. Med. **185,** 1481-1486.

Lockshin, R.A., Z. Zakeri, 2004: Apoptosis, autophagy, and more. Int. J. Biochem. Cell Biol. **36,** 2405-2419.

Madekurozwa, M-C, 2007: Ultrastructural features of the uterus in the sexually immature ostrich (Struthio camelus) during periods of ovarian inactivity and activity. Ondertepoort J. Vet. Res. **74,** 209-216.

McCowan, B., J. Schrader, A.M. Di Lorenzo, C. Cardona, D. Klingborg, 2006: Effects of induced molting on the well being of egg-laying hens. J. Appl. Ani. Welfare Sci. **9,** 9-23.

Parr, E.L., H.N. Tung, M.B. Parr, 1987: Apoptosis as the mode of uterine epithelial cell death during embryo implantation in mice and rats. Biol. Reprod. **36,** 211-225.

Reeder, R.L., B. Shirley, 1999: Deciliation in the ampulla of the rat oviduct and

effects of estrogen on the process. J. Exp. Zool. **283,** 71-80.

Ricke, S.C., 2003: The gastrointestinal tract ecology of *Salmonella enteritidis* colonization in molting hens. Poult. Sci. **82,** 1003-1007.

Ruszler, P.L., 1998: Health and husbandry considerations of induced molting. Poultr. Sci. **77,** 1789-1793.

Sandow, B.A., N.B. West, R.L. Norman, R.M. Brenner, 1979: Hormonal control of apoptosis in hamster uterine luminal epithelium. Am. J. Anat. **156,** 15-36.

Sato, T., Y. Fukazawa, H. Kojima, M. Enari, T. Iguchi, Y. Ohta, 1997: Apoptotic cell death during the estrous cycle in the rat uterus and vagina. Anat. Rec. **248,** 76-83.

Solomon, S.E., 1975: Studies on the isthmus region of the domestic fowl. Br. Poult. Sci. **16,** 255-258.

Spornitz, U.M., B.P. Rinderknecht, A. Edelmann, B. Scheidegger, F. Cairoli, 1994: Ultrastructure as a basis for dating of rat endometrium. Anat. Rec. **238,** 163-176.

Sundaresan, N.R., D. Anish, K.V.H. Sastry, V.K. Saxena, J. Mohan, K.A. Ahmed, 2007a: Cytokines in reproductive remodelling of molting white leghorn hens. J. Reprod. Immunol. **73,** 39-50.

Sundaresan, N.R., D. Anish, K.V.H. Sastry, V.K. Saxena, J. Mohan, M. Saxena, 2007b: Differential expression of lipopolysaccharide induced TNF-a factor (LITAF) in reproductive tissues during induced molting of white leghorn hens. Ani. Reprod. Sci. **102,** 335-342.

Sundaresan, N.R., D. Anish, K.V.H. Sastry, V.K. Saxena, K. Nagarajan, J. Subramani,

M.D.M. Leo, N. Shit, J. Mohan, M. Saxena, K.A. Ahmed, 2008: High doses of dietary zinc induce cytokines, chemokines and apoptosis in reproductive tissues during regression. Cell Tiss. Res. **332,** 543-554.

Thompson, E.B., 1994: Apoptosis and steroid hormones. Mol. Endo. **8,** 665-673.

Verhage, H.G., M.K. Murray, R.A. Boomsma, R.A. Rehfeldt, R.C. Jaffe, 1984: The postovulatory cat oviduct and uterus: correlation of morphological features with progesterone receptor levels. Anat. Rec. **208,** 521-531.

Verma, V., 1983: Ultrastructural changes in human endometrium at different phases of the menstrual cycle and their functional significance. Gynecol. Obstet. Invest. **15,** 193-212.

Walther, T.C., R.V. Farese, 2009: The life of lipid droplets. Biochim. Biophys. Acta., **1791,** 459-466.

Webster, A.B., 2003: Physiology and behaviour of the hen during induced molt. Poult. Sci. **82,** 992-1002.

Wick, R., A. Kress, 2002: Ultrastructural changes in the uterine luminal and glandular epithelium during the oestrous cycle of the marsupial *Monodelphis domestica* (grey short-tailed opossum). Cells Tiss. Organs. **170,** 111-131.

Yu, J.Y.L., R.R. Marquardt, 1974: Hyperplasia and hypertrophy of the chicken (*Gallus domesticus*) oviduct during a reproductive cycle. Poult. Sci. **53,** 1096-1105.

Zeiss, C.J., 2003: The apoptosis-necrosis continuum: insights from genetically altered mice. Vet. Pathol. **40,** 481-495.