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Transit along the vas deferens results in a high percentage of filiform spermatozoa with compacted chromatin in the rooster (*Gallus domesticus*)

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ABSTRACT

The present work aimed to evaluate the chromatin compaction of rooster spermatozoa along the male reproductive tract, and to study the vas deferens lining cells, potentially involved in sperm maturation. Chromomycin A3 (CMA3) was used to determine the chromatin compaction of spermatozoa from testis (T), proximal (including epididymis, V1), intermediate (V2) and distal (V3) vas deferens, and ejaculate (E). Six Birchen Leonesa roosters were used. E was obtained *in vivo* by dorso-ventral massage. V1, V2 and V3 sperm were obtained *post mortem* (six pairs of vasa deferentia), by flushing. T was obtained by washing the testes, cut in halves. The fixed cells were stained with CMA3 and propidium iodide for flow cytometry assessment. Results showed higher ($P < 0.01$) median fluorescence intensity (lower chromatin compaction) of T (693.8 ± 30.2) than V1 (546.3 ± 17.7), V2 (515.1 ± 12.1), V3 (517.6 ± 12.3) and E (491.4 ± 16.1). Regarding the percentage of intensely stained cells, T differs ($P < 0.05$) from V2, V3 and E, V1 differs ($P < 0.05$) from V3 and E, while V2, V3 and E do not differ. The histological analysis revealed secretory capacity of the vas deferens. Our findings specified that the transit though the vas deferens results in high percentage of compacted chromatin spermatozoa in E.

Keywords: chromatin condensation, flow cytometry, histology, protamines, rooster sperm chromatin, rooster sperm decondensation, sperm maturation process, vas deferens.

Introduction

It is known that, once formed, spermatozoa of most eutherian mammals undergo a maturation process in the epididymis, necessary to obtain motility and fertilisation capacities (Toshimori 2003; Cornwall and Horsten 2007; Robaire and Hinton 2015). Thus, post-testicular sperm maturation is completed by the time spermatozoa reach the cauda epididymis (Bauer *et al.* 2005). In the domestic fowl, spermatozoa undergo a maturation process through which the motility and fertilising capacities are acquired (Munro 1935; Etches 1996). However, it has been reported (Munro 1935, 1938; Howarth 1983; Ahammad *et al.* 2011*a*, 2011*b*; González-Santos *et al.* 2019) that fowl sperm maturation is not completed in the epididymis; instead, it continues along the passage of the spermatozoa through the vas deferens where they spend a number of hours up to some days depending on the species and conditions of life (an expected fact given the small size of the epididymis that is quickly passed by spermatozoa) (Munro 1935). Several studies on fowl sperm maturation have shown morphological, biochemical and/or physiological changes along the vas deferens (Munro 1935, 1938; Howarth 1983; Ahammad *et al.* 2011*a*, 2011*b*; González-Santos *et al.* 2019). The first studies on the functional maturation process of fowl spermatozoa were reported by Munro (1935, 1938). According to these studies, the motility in chicken spermatozoa is minimal in the testis, increases in the epididymis and maximises in the vas deferens. Regarding the fertilising capacity, Munro (1935) demonstrated, by intravaginal

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insemination in hens, that spermatozoa recovered from the testis, epididymis and vas deferens regions have different fertilising capacities, correlating with the capacity of movement. However, the intramagnal insemination of spermatozoa taken from testis, epididymis and vas deferens performed by Howarth (1983) resulted in a fertility rate of 85–90% during the first week after single insemination, suggesting that the maturation process in chicken spermatozoa through the male reproductive tract was mainly related to the acquisition of motility. More recent works about the functional competence acquired by fowl spermatozoa during their passage through different sections of the vas deferens (proximal, intermediate and distal) demonstrated a maturation process related to survivability (Ahammad *et al.* 2011*a*), and fertilising capacity as acrosomal proteolytic activity, penetrability of perivitelline layer (Ahammad *et al.* 2011*b*), acrosomal reaction capacity (González-Santos *et al.* 2019), protein binding capacity and acquisition of plasma membrane glycoproteins (Esponda and Bedford 1985; Morris *et al.* 1987). Moreover, other works demonstrated that fowl sperm morphology undergoes significant changes along the vas deferens (Nicander and Hellström 1967; González-Santos *et al.* 2019).

While the aforementioned competencies are all necessary to achieve the ability of fowl spermatozoa to fertilise the oocyte, the correct chromatin organisation ensures the integrity of DNA at the time of fertilisation, an essential feature for normal embryonic development and prevention of genetic alterations in the offspring (Fernández-Gonzalez *et al.* 2008). The transformation of the round spermatids into spermatozoa (spermiogenesis) is accompanied by the replacement, in the nuclei, of histones to protamines, leading to a highly packaged chromatin and cessation of transcription in mature spermatozoa (Mezquita and Teng 1977; Oliva and Dixon 1991). Protamines are small arginine-rich nuclear proteins that replace the histones in order to achieve a high level of chromatin compaction (Bizzaro *et al.* 1998). The main cause of condensation of sperm nuclei is the electrostatic interaction between the abundant arginine residues present in protamines and the anionic groups of DNA. This interaction has been proposed (Chiva *et al.* 1987) to be regulated *in vivo* through reversible post-transcriptional modifications such as enzymatic phosphorylation and dephosphorylation of serine residues (Louie and Dixon 1972). Most eutherian mammalian protamines possess cysteine residues, which allow stabilisation by formation of cysteine–cysteine disulphide bridges during the passage through the epididymis (Bedford and Calvin 1974; Mardani *et al.* 2014), and endow the sperm head with a rigidity that is believed to play a role in the zona pellucida penetration (Bedford and Calvin 1974). However, the galline protamine reported in roosters lacks cysteine (Oliva and Mezquita 1986; Rhim *et al.* 1995; Ausió *et al.* 2014). It has been suggested that, upon phosphorylation, serine and threonine residues of chicken protamine could form electrostatic links and therefore function like disulphide bonds (Chiva *et al.* 1987). The short avian epididymis and rapid sperm migration through it (Wishart and Horrocks 2000), together with the lack of cysteine in the fowl protamine identified so far, might suggest a more limited chromatin compaction process.

Chromomycin A3 (CMA3) is a specific fluorochrome used to identify poor chromatin condensation by indirectly measuring the level of sperm protamine deficiency. Moreover, CMA3 accessibility differs during spermiogenesis in mouse, in which testicular spermatids are highly CMA3 positive while mature spermatozoa are completely negative (Bianchi *et al.* 1993). Two models have been postulated to explain how the binding of CMA3 relates with the lack of protamines. The first one proposes that CMA3 and protamines both compete for the same binding site of DNA (Bianchi *et al.* 1993; Banaszewska *et al.* 2015). On the other hand, it has been reported that protamines bind to the major groove of the DNA, inducing conformational changes in the B-form of DNA, causing less effective binding of CMA3 to the minor groove of DNA (Bizzaro *et al.* 1998).

To the best of our knowledge, no information exists regarding the chromatin compaction process through the reproductive tract of male birds. The present study focuses on monitoring the compaction status of DNA, by chicken protamine, in different sections of the reproductive tract: proximal (V1, including epididymis), intermediate (V2) and distal (V3) sections of vas deferens, spermatozoa from testis (T) and ejaculated spermatozoa (E). In addition, the histological characteristics of different parts of the rooster reproductive tract were also analysed.

Materials and methods

Animals used in this work were handled according to procedures approved by the INIA Ethics Committee (Reference number PROEX 170/17) and were performed in accordance with the Spanish law of animal protection RD53/2013 which is in accordance with the European Union Directive 2010/63/UE.

Experimental birds

Six Birchen Leonesa roosters were used to analyse the sperm chromatin packaging. In addition, five more roosters were used for preparation of the positive and negative controls for flow cytometry. All birds were 2 years old and reproductively active. They belonged to the INIA's genetic resources conservation program (Campo 1998) and were kept in a 12 m^2 sand-floor pen under natural photoperiod, being fed with commercial feed containing 16% CP, 2700 kcal of ME/kg, 3.5% Ca and 0.5% available P over their entire life.

Experimental design

One week before euthanasia, ejaculates from trained roosters were obtained *in vivo*. Sperm samples were collected *post-mortem* from testes, and from three sections of the vas deferens (V): proximal (including epidydimis) (V1), intermediate (V2) and distal (V3). The different sperm samples were analysed to determine their morphology and the level of chromatin compaction (by CMA3). The different sections of V were fixed and analysed histologically to identify the different cellular types present in each section.

Collection of ejaculated spermatozoa

Ejaculated semen (E) was collected in 5 mL polystyrene tubes (Deltalab, Barcelona, Spain) by dorso-ventral massage (Burrows and Quinn 1937). Motility was subjectively assessed and scored, by a phase contrast microscope (Axiostar Plus, Zeiss, Jena, Germany), on a scale ranging from 0 to 5, as described by Santiago-Moreno *et al.* (2011), showing motility values corresponding to what is needed for fertilisation (≥90%, score 4–5). The E was then diluted 1:1 (v/v) in Lake–Ravie medium at room temperature (Lake and Ravie 1984) composed of sodium glutamate (102.6 mM), glucose (40.4 mM), magnesium acetate (3.73 mM), potassium acetate (50.9 mM), polyvinylpyrrolidone (M_r 10 000; 0.3 mM) and 100 mL H_2O (final pH 7.08, final osmolality 343 mOsm/kg; hereinafter referred to as LR84). The total volume was then transferred to a 1.5-mL Eppendorf tube for subsequent fixation (see sperm preparation).

Post mortem sperm collection

Animals were killed by an injection of 40 mg of Embutramide i.v (T-61 euthanasic, Intervet International GmbH, Freising, Germany) per kilogram of weight. Testes with their corresponding vas deferens were extracted as described by Villaverde-Morcillo *et al.* (2016). Vas deferens were separated from the testis and divided in three same length sections: proximal (V1, including epididymis), intermediate (V2) and distal (V3) sections. The semen contained in each section was then collected by the flushing method described by Villaverde-Morcillo *et al.* (2016) with minor modifications. Thus, 1 mL of LR84 at room temperature was injected with a 25G needle into the proximal extreme of each section of the vas deferens (V1, V2 and V3) and collected from the distal extreme in a 1.5 mL microtube.

Each testis was dissected in two parts and washed with 1 mL of LR84. Then the washing medium containing the testis spermatozoa (T) was transferred to a 1.5 mL microtube.

Hemacolor staining

Morphology of the cells was determined after fixation and staining by Hemacolor (Merck KGaA, Darmstadt, Germany). In brief, 5 μL of the collected spermatozoa (E, V1, V2, V3 or T) were smeared onto glass slides and left to air-dry. Each sample was prepared by duplicate. The fixed smears were consecutively incubated with the acid and alkaline staining solutions for 2 min each (Villaverde-Morcillo *et al.* 2015). Once stained and dried, all slides were sealed with Eukitt mounting medium (Panreac Quimica S.L.U., Barcelona, Spain) and a coverslip. The smears were analysed by bright field microscopy (Motic BA210, Motic, Barcelona, Spain) at 1000×. The cells found in each smear were classified in three groups: round cells (R), spermatids with rests of cytoplasm (S + C) and filiform spermatozoa (F). The percentage of each group was determined.

Sperm preparation

For the analysis of chromatin packaging 50 μL of E diluted in LR84 were mixed with 450 μL of 4% formaldehyde in PBS and kept at 5°C for at least 30 min for fixation. T and V sperm samples were centrifuged (500*g*, 10 min) and the supernatant discarded. The cells from the pellet were fixed by resuspension in 500 μL (or 1 mL for T) of 4% formaldehyde in PBS and kept at 5°C for at least 30 min. Once fixed, samples (E, T and V) were washed twice by centrifugation (1300*g*, 10 min) with 500 μL and 200 μL PBS for the first and second washes, respectively. T pellets were then resuspended for permeabilisation in 500 μL of iced-cold 70% ethanol while E, and V pellets were resuspended in 200 μL (100 μL for scarce pellet). All samples were stored at −20°C until staining with CMA3. Concentration of the stored samples was determined employing the CASA system, previous dilution in PBS containing BSA (10 mg/mL) (PBS-BSA). The CASA system (Sperm Class Analyser, SCA v.4.0 software, Microptic S.L., Barcelona, Spain) was coupled to a phase contrast microscope (Nikon Eclipse model 50i; negative contrast) (Santiago-Moreno *et al.* 2012).

Preparation of control samples

To validate the CMA3 staining method in rooster spermatozoa we prepared two controls (Fig. 1): (i) ejaculated spermatozoa showing high chromatin packaging (negative control) and (ii) deprotaminised ejaculated spermatozoa (positive control). For the negative control, a pool of ejaculates from five roosters was prepared as described above. For the positive control another pool of the same five roosters followed a treatment for chromatin decondensation by protamines displacement used in mice and human spermatozoa (Bizzaro *et al.* 1998), which we modified (regarding incubation times, fixation and permeabilisation procedure) to make it effective in rooster sperm. Briefly, the sperm pool was divided in fractions of 100 μL and the seminal plasma was removed by centrifugation (170*g*, 10 min). The resultant pellet was washed twice with 200 μL PBS-BSA (170*g*, 10 min), resuspended and

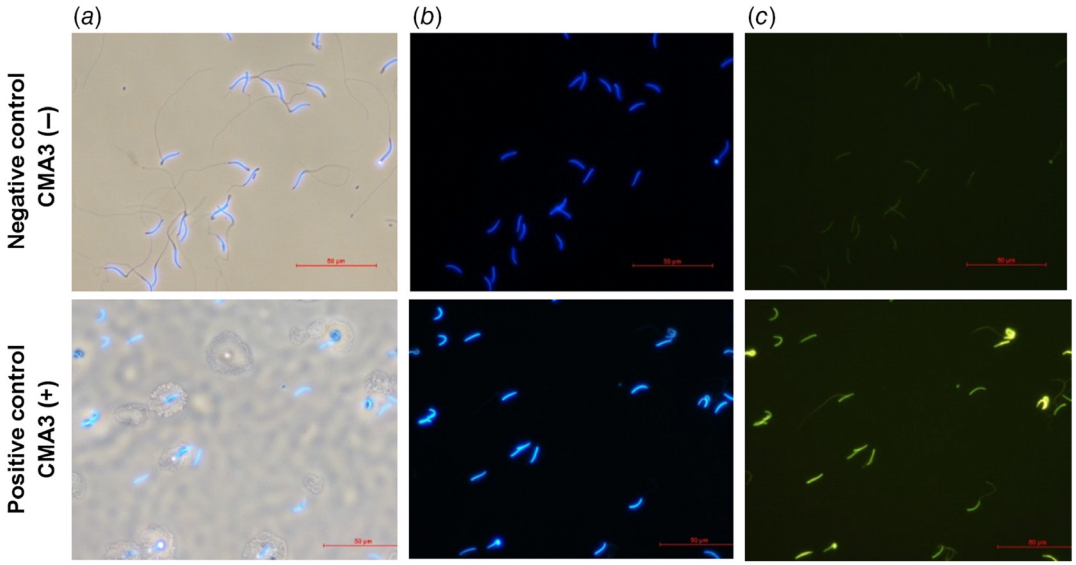

Fig. 1. Negative control (ejaculated spermatozoa) and positive control (deprotaminised ejaculated spermatozoa) of CMA3 fluorescence observed by epifluorescence microscopy. (*a*) Controls observed under bright field and UV light. (*b*) Controls observed under UV light for excitation of Hoechst 33342 and detection of the DNA present in the sample. (*c*) Controls observed under 488 nm wavelength for excitation of CMA3 and detection of chromatin compaction: the more yellow, the less grade of compaction. The red line corresponds to 50 μm.

incubated at room temperature for 24 h in 200 μL of PBS containing 0.1% Triton X-100 and 5 mM dithiothreitol (DTT). After two washes with 200 μL of PBS-BSA and two washes with PBS (500*g*, 15 min), the pellet was resuspended and fixed with 200 μL of ice-cold 1% formaldehyde. Following 30 min of incubation at 5°C, 150 μL of 4% formaldehyde were added twice in 5 min interval. The formaldehyde was then removed (1300*g*, 20 min) and the pellet washed once with 200 μL of PBS and once with 200 μL of PBS-BSA (1300*g*, 20 min). Pellet was then resuspended and incubated for 24 h in 200 μL of 1 M NaCl and 5 mM DTT in distilled water. The cell suspension was centrifuged (1300*g*, 20 min) and supernatant discarded. The resultant pellet was washed (1300*g*, 20 min) with 100 μL of PBS-BSA followed by another washing with 100 μL of McIlvaine (see below) (3000*g*, 20 min). The sperm pellet was then resuspended with 100 μL of ethanol and store at −20°C until staining with CMA3 (within 1 week). Despite the preparations used for the sperm deprotamination contained DTT (as described by Bizzaro *et al.* 1998), this component could be removed for the case of rooster spermatozoa due to the absence of cysteine in the chicken sperm protamines. To confirm the deprotamination of spermatozoa, the deprotaminated sample (treated ejaculated) was observed, along with the negative control (non-treated ejaculate), under epifluorescence microscopy (Fig. 1). The staining procedure is described in the section named 'Analysis by epifluorescence microscopy'.

Analysis by flow cytometry

Stored samples in ethanol were transferred to each well of a 96-well cytometer plate and diluted in PBS-BSA (10 mg/mL) to obtain 200 μL of 3×10^6 spermatozoa/mL (all sperm samples and controls were analysed at the same time). The plate was centrifuged (1300*g*, 10 min) and supernatants discarded. Pellets were washed once with 200 μL of PBS-BSA (10 mg/mL) and then resuspended in 200 μL of McIlvaine buffer, adding 1 μL of CMA3 0.5 mg/mL (Ex/Em wavelenghts: 440/550 nm; Jacobsen and Jakobsen 1999) to each well. After 2 h of incubation samples were washed once (1300*g*, 10 min) and resuspended again with 200 μL of McIlvaine buffer ([82.4 mL of 0.2 M Na_2HPO_4 + 17.6 mL of 0.1 M citric acid] containing 10 mM $MgCl_2$, pH 7) (1300*g*, 10 min). The samples contained in each well were then transferred to cytometer tubes containing 200 μL of McIlvaine buffer and 1 μL of propidium iodide (Ex/Em wavelengths: 535/617 nm; https://tools.thermofisher.com/content/sfs/manuals/mp07011.pdf). The samples were incubated a minimum of 10 min before their analysis by flow cytometry.

Samples were analysed with a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). The SSC vs FSC dot plot allows identifying and gating the cell populations according to their size and complexity. Debris, round cells, spermatids and filiform spermatozoa counts on different size and complexity, each of these groups should occupy different regions on the dot plot. Taking this into

account we used ejaculated sperm as control to gate the filiform population. To determine the limiting value of fluorescence intensity for considering a spermatozoon as positive, non-treated ejaculated spermatozoa incubated with CMA3 (and propidium iodide) were used as control. Any spermatozoon with fluorescence intensity higher than this limit was considered as positive for CMA3. Deprotaminised spermatozoa stained with CMA3 were used as positive control to validate the limit established with the negative control. Detection of propidium iodide was performed by the blue laser (488 nm) and the Filter 2 (PE-A filter, 585/42 nm). A total of 10 000 PI positive gated events were acquired per determination. Regarding the CMA3 detection, the violet laser (405 nm) and the Filter 8 (Amcyan filter, 502/25 nm) were used. Acquired data were analysed using FlowJo software (Becton-Dickinson, Franklin Lakes, NJ, USA). The % of population CMA3(+) and its median fluorescence intensity (MFI) were calculated from each sample by the flow cytometer.

Analysis by epifluorescence microscopy

Samples were diluted in PBS-BSA (10 mg/mL) to obtain 200 μL of 10×10^6 spermatozoa/mL. The samples were washed following the same procedure as for flow cytometry and finally were resuspended in 200 μL of McIlvaine containing 1 μL of CMA3 (0.5 mg/mL). After 30 of incubation, 4 μL of ProLong Antifade Reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and 10 μL of Hoechst 33342 (1 mg/mL) were added and left for incubation 30 min, then, samples were immediately observed on slides covered with 0.02% polylysine. Each sample was analysed under a Nikon Eclipse E200 epifluorescence microscope at 400× employing the wavelengths of 450–490 nm for excitation of CMA3 (emission 575 nm, yellow) and 330–380 nm for excitation of Hoechst 33342 (emission 461 nm, blue). The differences in incubation times between cytometer and epifluorescence analysis was determined by the time required to objectively discern between the negative and positive controls. For epifluorescence microscopy, one hour of sperm incubation with CMA3 (30 min in presence of CMA3 + 30 min in presence of CMA3 and Hoechst 33342) was necessary to visually differentiate the positive control from the negative (Fig. 1). However, the analysis by flow cytometer showed that the CMA3 staining could have subtle changes with longer incubation times, which could not be appreciated by epifluorescence microscopy. As we anticipated that the spermatozoa retrieved from the different parts of the reproductive tract were could have (great or subtle) differences in staining intensities, we determined (based on previous incubation assays of 1 h, 2 h, 3 h, unpubl. data) that 2 h was the optimal incubation time to determine the minimal value of a sperm fluoresce intensity to consider an spermatozoon as positive for CMA3.

Histological typing of the vas deferens

The vas deferens tissues, fixed in 10% buffered formalin were dehydrated in a grade alcohol series according to routine histological technique and embedded in Paraplast Plus™ (Leica Biosystems, Wetzlar, Germany). Histological sections (5 μm thick) were stained with hematoxylin and eosin and were observed under bright field microscope (Motic BA210, Motic Spain) to characterise their histological appearance.

Statistical analysis

The sperm variables were not normally distributed as determined by Shapiro–Wilk test, even after arcsine and log transformations. Thus, nonparametric analyses were used. Kruskal–Wallis analysis and a 'Multiple comparisons of mean ranks for all groups' were used to compare differences between the different sections of the vas deferens including testes and ejaculate (Siegel and Castellan 1995; Fowler *et al.* 2009). Data were expressed as means ± s.e.m. All statistical calculations were made using TIBCO Statistica™ software v.13.3 (TIBCO Software Inc., Palo Alto, CA, USA). Statistical differences were considered significant when $P < 0.05$.

Results

Morphology of the cells obtained from different sections of the rooster reproductive tract

The morphology of the cells obtained from the different sections of the vas deferens was analysed and compared with the cells of the testis and ejaculate (Fig. 2). Three kinds of cell populations were found: round cells (R), spermatids with cytoplasm (S + C) and filiform spermatozoa (F). As can be seen in Fig. 2 the R population was significantly more abundant ($P < 0.05$) in testis (44.6% ± 3.8) than any of the other sections and the ejaculate (0%), moreover it was the most abundant population present in testis spermatozoa. Regarding S + C, testis showed the highest population (29.2% ± 3.7), being negligible (<0.5% ± 0.1) for the other tract sections and the ejaculate. Regarding F, testis showed significant differences ($P < 0.05$) with the other parts of the male tract, exhibiting the lowest values (25.8% ± 3.6) vs values higher than 90.9% ± 1.7 for the other sperm samples. Significant differences ($P < 0.05$) were seen between V1 (90.9% ± 1.7) and V2 (93.8 ± 1.7) vs E (100%) while V3 (95.1 ± 1.8%) did not differ from E (100%).

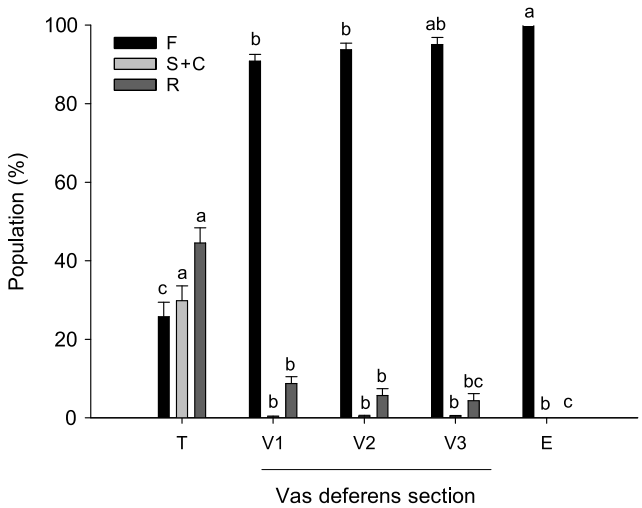

Fig. 2. Morphology analysis of rooster (sperm) cells, obtained from testis, different sections of vas deferens and ejaculate. The spermatozoa were stained by Hemacolor and observed under bright field microscopy. F, filiform spermatozoa; S + C, spermatids with rests of cytoplasm; R, round cells; T, testis; V1, proximal vas deferens; V2, intermediate vas deferens; V3, distal vas deferens; E, ejaculate. Different letters (a, b and c) indicate significant differences ($P < 0.05$) between sperm samples belonging to the different parts of the tract and the ejaculate. Data are shown as mean ± s.e.m.

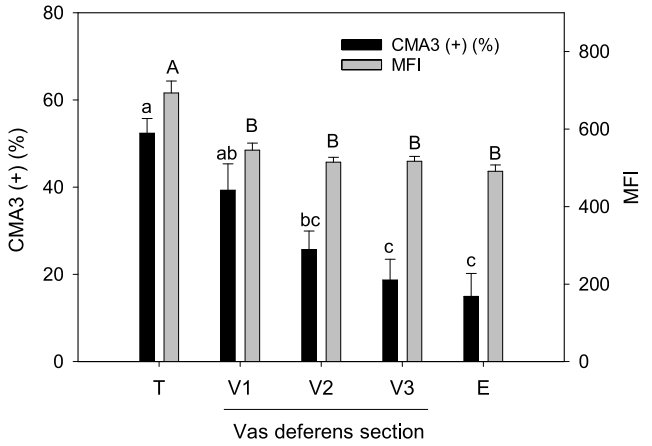

Fig. 3. Percentage of CMA3 (+) and median fluorescence intensity (MFI), evaluated by flow cytometry, of rooster filiform spermatozoa from different sections of the vas deferens, including testis and ejaculate. T, testis; V1, proximal vas deferens (including epididymis and a more distal section); V2, intermediate vas deferens; V3, distal vas deferens; E, ejaculate; CMA3 (+), positive in chromomycin A3. Different letters (a, b, c; A, B) indicate significant differences ($P < 0.05$; $P < 0.01$, respectively) between sperm samples belonging to the different parts of the tract and the ejaculate. Data are shown as mean ± s.e.m.

Chromatin compaction of the cells obtained from different sections of the rooster reproductive tract

Flow cytometry allowed assessing the ability of CMA3 to access the DNA of filiform spermatozoa. The results obtained are described in Fig. 3. The decrease in the median fluorescence intensity (MFI) of CMA3 corresponds to an increase in chromatin compaction, thus spermatozoa showing intense fluorescence (CMA3+) correspond to spermatozoa with no compacted chromatin. Results showed a decline ($P < 0.01$) in the median fluorescence intensity (MFI) of T (693.8 ± 30.2) vs V1 (546.3 ± 17.7), V2 (515.1 ± 12.1), V3 (517.6 ± 12.3) and E (491.4 ± 16.1). Regarding the percentage of CMA3 (+) filiform spermatozoa i.e. of the filiform spermatozoa with intense fluorescence detected by the cytometer, T differed ($P < 0.05$) from V2, V3, E (but not from V1), V1 differed ($P < 0.05$) from V3 and E while V2, V3 and E did not differ. An example of the cytograms obtained can be seen in Fig. 4. As it can be observed there is a decrease in chromatin condensation from testis to the end of the vas deferens.

A comparison between the different variables analysed along the rooster tract (Fig. 5), i.e. % filiform spermatozoa (%F), % spermatozoa with cytoplasm (%S + C), % round cells (%R), the % of F being CMA3+ (CMA3+) as well as the MFI of F spermatozoa (MFI) show that a strong increase ($P < 0.05$) of %F in the passage from T to V1 coincided with a significant decrease in the MFI of F ($P < 0.01$), and a decrease of %S + C ($P < 0.05$) and %R ($P < 0.05$). A progressive decrease of intensity of %CMA3+ was observed from T to E.

Histological analysis in different sections of rooster vas deferens

As all the rooster showed homogeneity regarding motility characteristics of their ejaculates, the fixed tissues of one randomly chosen rooster were observed under bright-field microscopy (Figs 6, 7, 8 and 9). The proximal vas deferens section (including epididymis) was analysed at two positions, one close to the epididymis (position 1) and another distal from it (position 2). Section of position 1 showed numerous conduits (Fig. 6*a*). The conduits presented a great number of folds covered with simple pseudostratified epithelium (*p*). There were some conduits with a notably wider lumen (6*b*), possibly initiations of vas deferens. In sections of the more distal position on V1 (position 2, Fig. 6*c*), a broad folded duct was observed that was internally lined with simple pseudostratified epithelium (*p*, Fig. 6*d*). Yellow arrows of Fig. 6*d* indicate the presence of a kind of channels running in the direction of the lumen.

In the intermediate vas deferens (V2, Fig. 7*a*), the duct was wider. This was the only segment in which the presence of a simple coiled tubular gland was observed (Fig. 7*b*). As can be observed in more detail in images 7*c* and 7*d*, the vas deferens is internally lined with simple pseudostratified epithelium

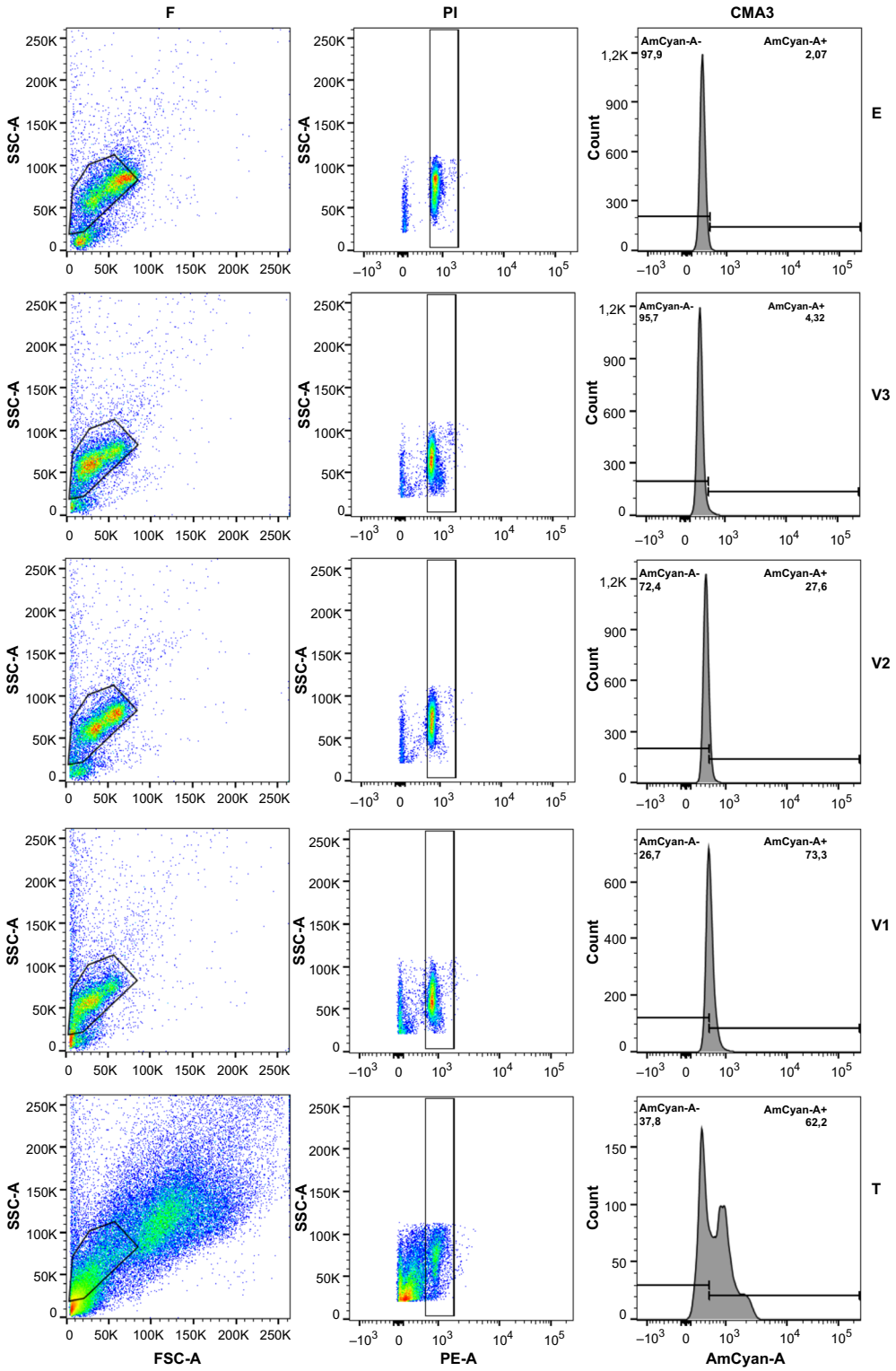

Fig. 4. Cytograms obtained from the analysis of spermatozoa belonging to different sections of one of the rooster reproductive tracts analysed. The cytograms correspond to the gating on a SSC/FSC dot plot of filiform spermatozoa (F), identified using ejaculated spermatozoa (E) as control; selection of singlets by staining with propidium iodide (PI); and quantification of the percentage of mature and immature sperm nuclei by staining with Chromomycin A3 (CMA3). Detection of PI (Ex/Em wavelenghts: 535/617 nm) was performed by the blue laser (488 nm) and the Filter 2 (PE-A filter, 585/42 nm). Regarding the CMA3 detection (Ex/Em wavelenghts: 440/550 nm), the violet laser (405 nm) and the Filter 8 (Amcyan filter, 502/25 nm) were used. K, thousands.

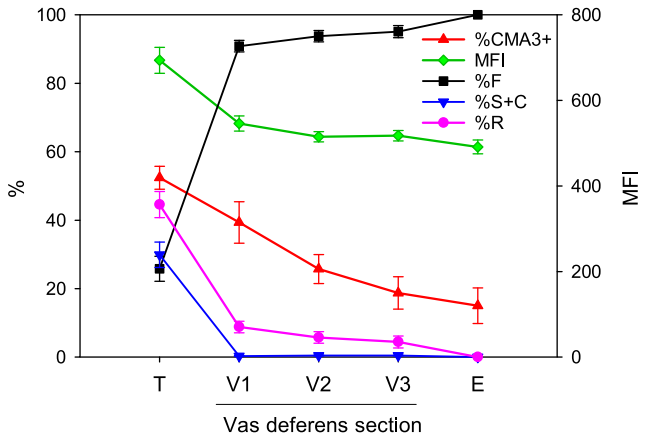

Fig. 5. Comparison between different variables analysed along the rooster male tract: % filiform spermatozoa (%F), % spermatids with rest of cytoplasm (%S + C), % round cells (%R), % of F CMA3+ (CMA3+), as well as the MFI of F spermatozoa (MFI). T, testis; V1, proximal vas deferens; V2, intermediate vas deferens; V3, distal vas deferens; E, ejaculate; MFI, median fluorescence intensity; CMA3+, positive in chromomycin A3. Data are shown as mean ± s.e.m.

with a smooth surface in some areas of the lumen (7*c*) and ridges and valleys in others (7*d*). In Fig. 7*c* the epithelium presents clear basal round cells (black arrow) intercalated between basophilic columnar cells (solid white arrows). In Fig. 7*d* clear basal round cells are also observed (black arrows) and other clear columnar cells (blue arrows), the latter are seen in the valleys, resembling intraepithelial glands (black box of dotted lines).

A more distal section of V2 (Fig. 8*a*) featured long protuberances with spermatozoa apparently accumulated in the valleys (Fig. 8*d*) while some protuberances seem to contain a kind of fluid with which spermatozoa appear to interact (Fig. 8*c*). Fig. 8*b* shows a ridge with the presence of clear basal round cells (black arrows) between basophilic columnar epithelial cells (white arrows), some of them with channels that approach to the lumen (*ch*); spermatozoa were not present. Fig. 8*e* shows that spermatozoa (*z*) concentrate in the channels of the ridge, possibly interacting with the cells that form it or with the invaginations/channels content.

Fig. 9 shows the distal section of vas deferens (V3) corresponding to the right (Fig. 9*a*) and left (Fig. 9*d*) testis. Despite de difference in size, both sections were found to share a similar structure. Both are internally lined with simple pseudostratified epithelium (Fig. 9*b, e* and *g*) with some sections forming ridges and valleys (Fig. 9*g*) and others smoother (Fig. 9*e*). In either type of section, sperm cells immersed in acidophylic secretion (*s*) were observed (Fig. 9*c, g* and *h*). Fig. 9*e–h* show that spermatozoa interact

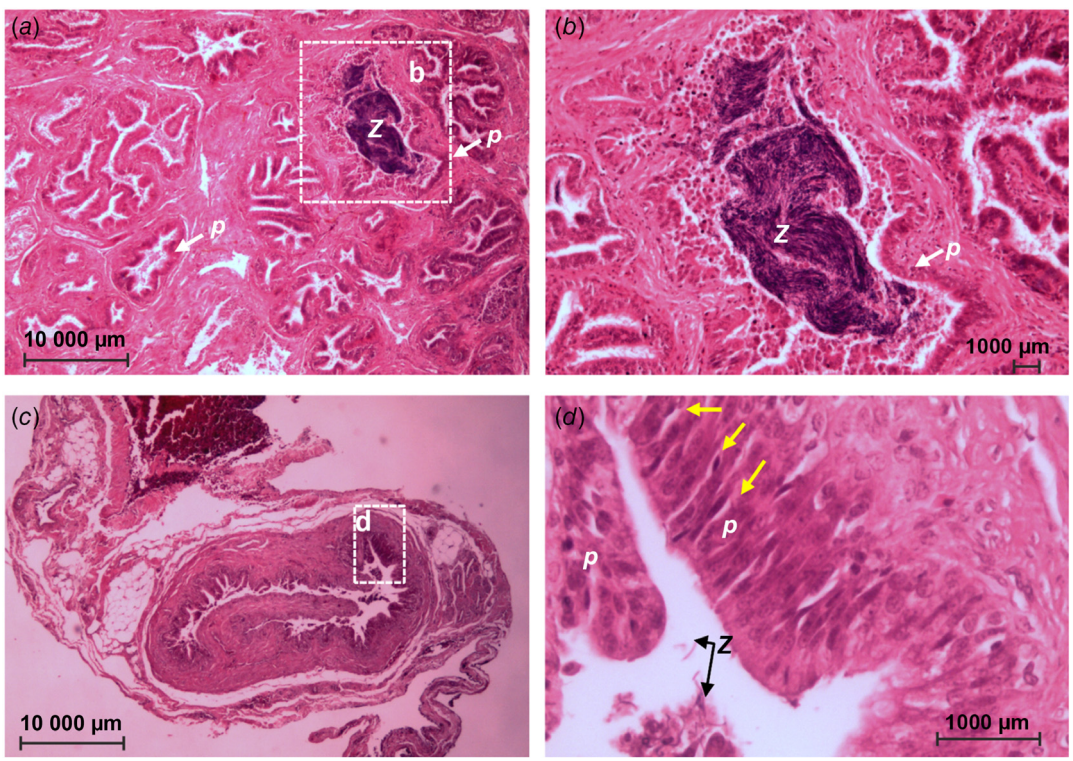

Fig. 6. Proximal section of rooster vas deferens (V1) stained by hematoxylin–eosin. (*a*) Proximal section of vas deferens close to the epididymis (position 1, 40×). (*c*) Proximal section of vas deferens, distal from epididymis (position 2, 40×). (*b*) and (*d*) are detailed images (100× and 400×, respectively) of (*a*) and (*c*). *p*, pseudostratified epithelium; *z*, spermatozoa. Yellow arrows, channels running in the direction to the lumen.

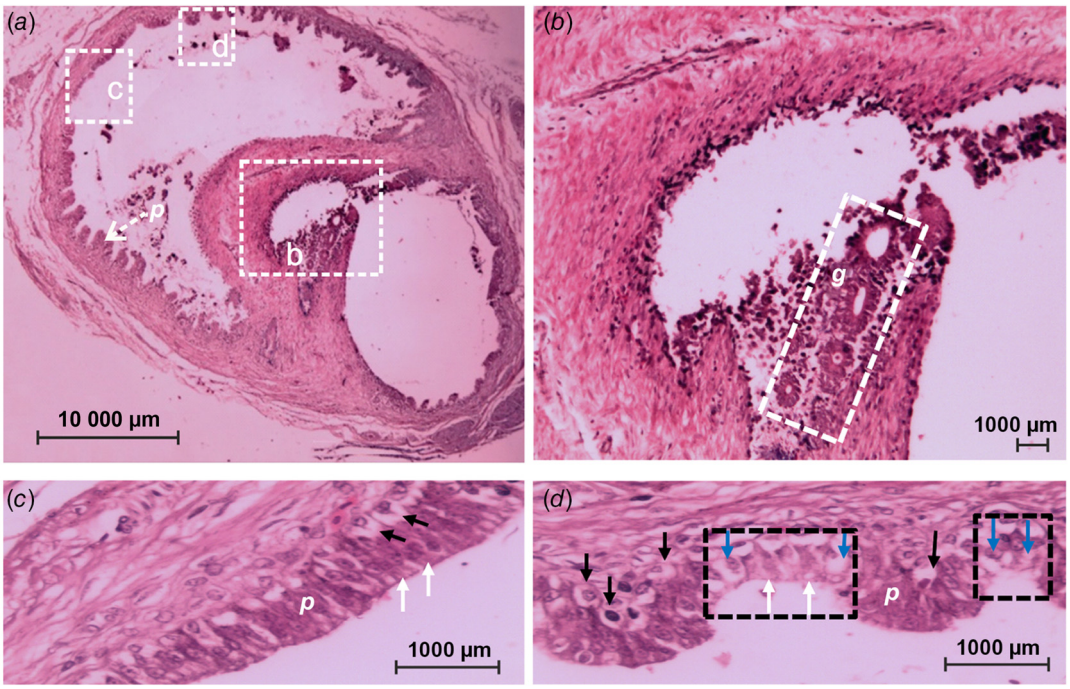

Fig. 7. Intermediate section of rooster vas deferens (V2), adjacent to the proximal vas deferens section (V1), stained by hematoxylin–eosin. (*a*) Image constructed with photographs at 40× taken from different parts of the same histological section of V2. (*b–d*) are detailed images (100×, 400× and 400×, respectively) of (*a*). *p*, pseudostratified epithelium; *z*, spermatozoa; *g*, coiled or folded tubular gland. Black arrows, clear basal round cells; solid white arrows, basophilic columnar cells; blue arrows, clear columnar cells in the valleys of the epithelium (possible intraepithelial glands), black dotted box, possible intraepithelial glands.

with or stick to the spaces where clear basal round cells are located.

Discussion

CMA3 has been used to identify protamine deficiency in spermatozoa of many species, such as human, bull, rat and rooster (Kazerooni *et al.* 2009; Simões *et al.* 2009; Mardani *et al.* 2014; Banaszewska *et al.* 2015), however, the protocols used involve the staining of spermatozoa in smears for subsequent analysis by epiflouorescence microscopy (Cortés-Gutiérrez *et al.* 2007). In the present study we successfully adapted a staining protocol with CMA3 that can be used in chicken to detect objectively and with high accuracy abnormal sperm chromatin condensation and its relation to fertility problems. The results obtained in the present study revealed that the transit of rooster sperm through the vas deferens resulted in an increase in the percentage of spermatozoa with compacted chromatin. This finding contributes along with other works (Munro 1935, 1938; Howarth 1983; Esponda and Bedford 1985; Morris *et al.* 1987; Ahammad *et al.* 2011*a*, 2011*b*; González-Santos *et al.* 2019) in the demonstration that vas deferens in rooster induces important physiological and structural modifications to produce mature and functional populations of spermatozoa, a similar role of epididymis in mammals. In this regard, Tingari (1971) in his studies on the structure and epididymal region and vas deferens of rooster spermatozoa concluded that avian epididymal region could be regarded as equivalent to the caput epididymidis of scrotal mammals while different regions of the vas deferens might be considered analogous to the corpus and cauda epididymis (Tingari 1971).

In the present study, we demonstrate a decrease in the filiform spermatozoa with no compacted chromatin along the vas deferens, indicating the importance of a compacted organisation of rooster chromatin before ejaculation, very probably related to motility and penetration capacity, besides its role in ensuring the integrity of DNA at the time of fecundation. Protamines have an effect on sperm shape (Czernik *et al.* 2016), and it has been demonstrated that both sperm shape and size characteristics are related with swimming velocity of spermatozoa (Malo *et al.* 2006). Regarding the morphology of the rooster sperm along the vas deferens, most spermatozoa are filiform once they leave testis. The percentage of round cells present in V1, V2 and V3 decreased along the male tract and were not observed in the ejaculates. Whether these cells correspond to desquamation of epithelial cells or cells with a role on sperm maturation is unknown, however, a study on resorption of

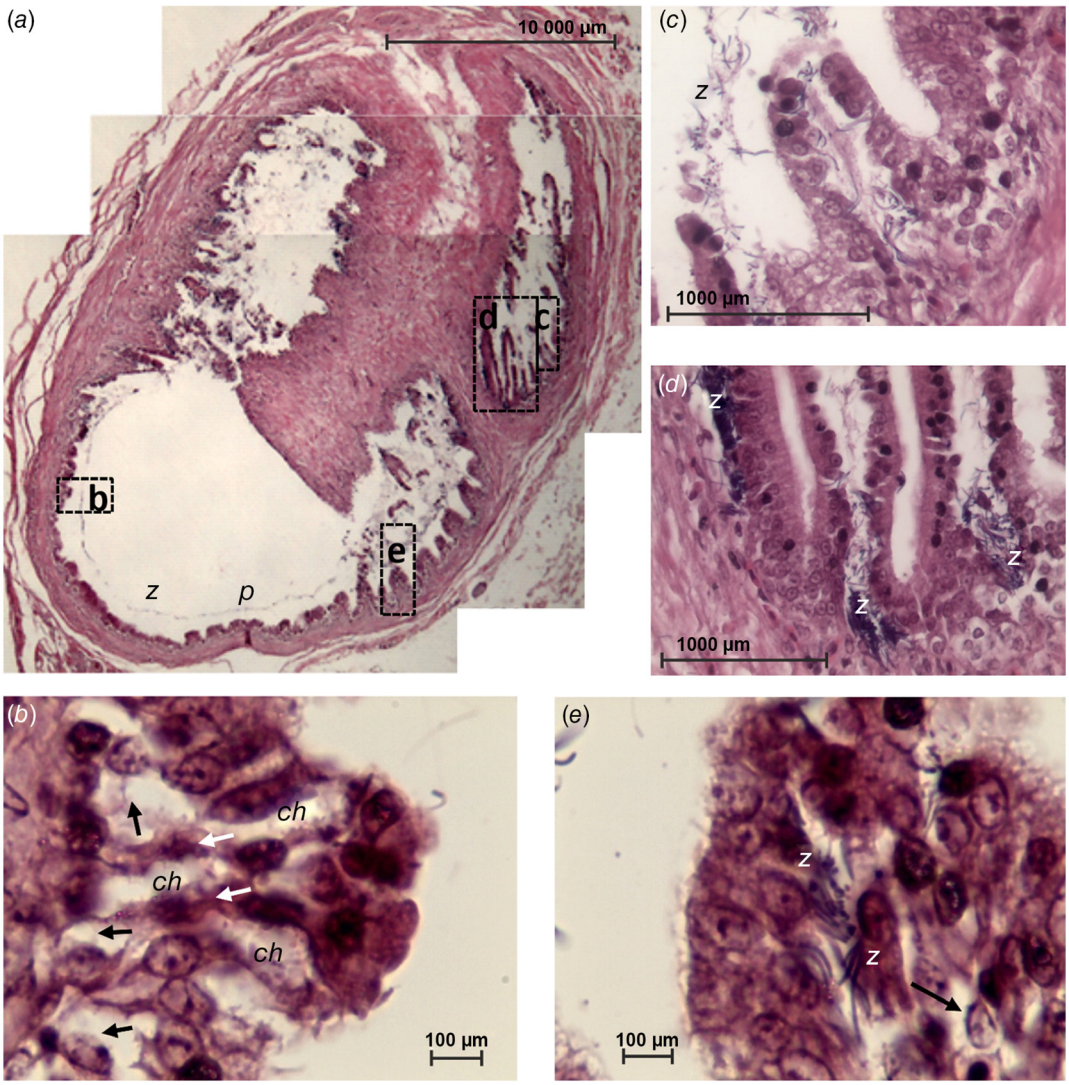

Fig. 8. Intermediate section of rooster vas deferens (V2), closer to the distal section of vas deferens (V3), stained with hematoxylin–eosin. (*a*) Image constructed with photographs at 40× taken from different parts of the same histological section of V2. (*b*–*e*) are detailed images (1000×, 400×, 400× and 1000x, respectively) of (*a*). *p*, pseudostratified epithelium; z, spermatozoa; *ch*, Black arrows, clear basal round cells.

spermatozoa in the rooster excurrent ducts, reported presence of free cells, debris and desquamated epithelia in roosters with normal reproductive tract (Tingari and Lake 1972). The presence of spermatids with cytoplasm in the vas deferens could be due to the management of the male tract, whether it is not the case, its absence in ejaculated spermatozoa could be due to the resorption of these cells by the vas deferens as we explain below.

The increase of filiform spermatozoa was coincident with the decrease in CMA3+ sperm and MFI in the passage from T to V1, indicating that the chromatin of most filiform spermatozoa of V1 is inaccessible to the probe. Although the MFI does not vary significantly along the rest of the sections, the percentage of CMA3(+) cells showed a progressive decrease to the distal part of the tract. We propose two possible reasons. First, the resorption by the rooster excurrent ducts of the spermatozoa (and/or other cells) with no proper chromatin organisation. This hypothesis builds on the observation of epithelial spermatophagy in the reproductive tract of avian species, such as chicken (Tingari and Lake 1972) and passerine birds (Chiba *et al.* 2011). While in passerine birds, the epithelial spermatophagy occurs in the seminal glomerula (Chiba *et al.* 2011), in chicken, it appears to occur in a wide range of the seminal tract, including the vas deferens (Tingari and Lake 1972). For chicken, Tingari and Lake (1972) reported three epithelial cell types responsible of sparmatophagy: the low cuboidal cells in the rete testis, the ciliated cell lining the efferent and connecting ducts in the epididymis and the non-ciliated type II cells in the vas

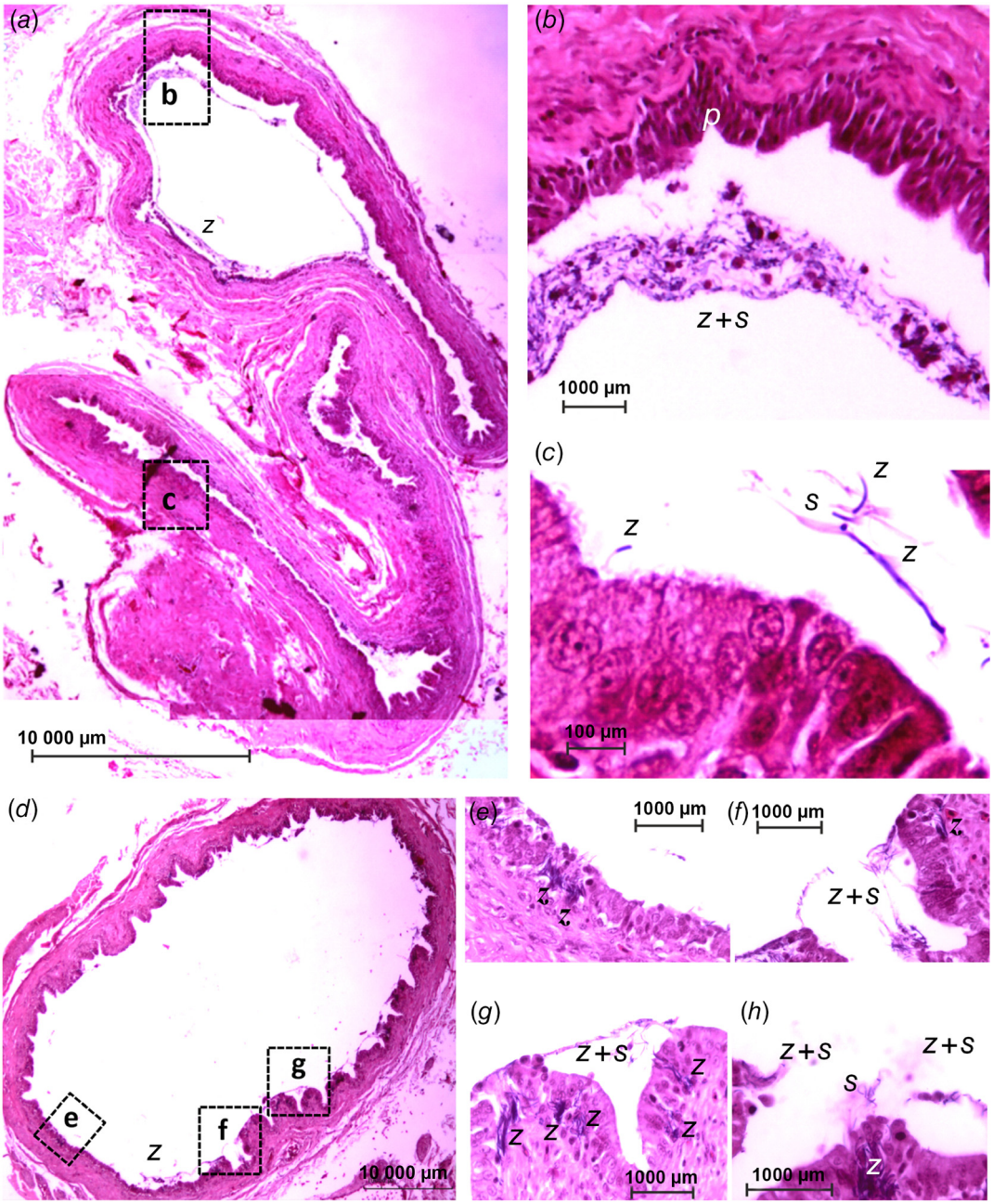

Fig. 9. Distal section of rooster vas deferens (V3), stained with hematoxylin–eosin. (*a*) Right vas deferens, closest to the cloaca. Due to the large size of the vas deferens, the image was constructed with photographs at 40× taken from different parts of the same histological section of V3. (*b*), and (*c*) are detailed images (100× and 1000×, respectively) of (*a*). (*d*) Initial part of the left V3 (40×). (*e–g*) are detailed images (400× all of them) of (*d*). (*h*). Photograph taken from another histological section of left V3 (400×). Pseudostratified *p*, pseudostratified epithelium; *z*, spermatozoa; *s*, acidophylic secretion.

deferens. On the other hand, Słowińska *et al.* (2020) reported that apoptosis may be primarily involved in the regulation of post-testicular sperm maturation quality in turkey. Therefore, the induction of apoptosis in rooster spermatozoa that do not have proper chromatin condensation along the vas deferens should not be ruled out. A second possibility is that rearrangement of the rooster sperm chromatin continues in the vas deferens by phosphorylation of protamines as it happens in the testes (Słowińska *et al.* 2020). Although it is assumed that chromatin condensation of mammalian spermatozoa is completed when they reach the cauda of the epididymis (which is well-developed in mammals), Bauer *et al.* (2005) discovered that the spermatozoa of the eutherian mammal spinifex hopping mouse (*Notomys alexis*)

spermatozoa do not finish the maturation process in the epididymis but undergo chromatin compaction in the vas deferens (Bauer *et al.* 2005), probably due to the short size of the epididymis which results in a rapid epididymal transit; a shared characteristic with birds. Moreover, unlike most mammalian species, in which spermatozoa remain stored in the epididymis, the spinifex hopping mouse showed some sperm storage in the distal region of the vas deferens (as observed in birds), where chromatin with higher resistance to decondensation was found than in cauda epididymis. These coincidences between the spinifex hopping mouse and avian species, support the fact that fowl sperm maturation continues along the passage of the spermatozoa through the vas deferens (Munro 1935, 1938; Howarth 1983; Ahammad *et al.* 2011*a*, 2011*b*; González-Santos *et al.* 2019, among others), including a process of sperm chromatin condensation as suggested by our findings. The chromatin condensation process in birds differs from mammals' spermatozoa, but organisation is structurally stable despite the lack of disulphide bridges. In fact, the cysteine positions in mammalian protamines are either substituted by serine or threonine in birds (Chiva *et al.* 1987). In ostrich, the main protamine component P-II reveal structural and compositional characteristics intermediate between Neognathae birds and reptiles. Ostrich present high content in histidine (Ausió *et al.* 1999), similarly to reptiles such as crocodilians and lizards. The presence of histidine residues has led to hypothesise that zinc, abundant in the spermatozoa of both invertebrates and vertebrates (Ausió *et al.* 1999), may bind to some of the histidine residues as occurs in the mammalian histidine/cysteine residues of P2 protamines. This could play a role in the way these proteins bind to DNA, possibly through zinc fingerlike motifs (Gatewood *et al.* 1990; Bianchi *et al.* 1992).

Previous studies with the proximal, intermediate and distal vas deferens (Ahammad *et al.* 2011*a*, 2011*b*; González-Santos *et al.* 2019) demonstrated an increase in capabilities such as motility, survivability, acrosome proteolytic activity, acrosomal reaction capacity, penetrability of the inner perivitelline, as the spermatozoa approximate to the distal section. It may be that specialised cells in the lining of the vas deferens, or their secretions, play a role in these maturation changes. The histological analysis of the three different sections of the rooster vas deferens that we made (V1, V2 and V3) showed the presence of pseudostratified columnar epithelium. In their work, Razi *et al.* (2010) mentioned that distally, the epithelium of the ductus deferens changed to a simple cuboidal type but we did not observe this in the sections analysed in our study (Razi *et al.* 2010). This might be due to differences between the individuals analysed or to tissue sections taken at different distances from the cloaca.

In our study we found an abundance of two types of foamy cells in the V2 section: one type, located on the basal line of the epithelium (clear basal round cells), some of which formed a kind of channel that extended to the lumen without reaching it at all, and another type that was exposed to the lumen, resembling intraepithelial glands (clear columnar cells in the valleys). The foamy structure of these cells suggests their secretory capacity, which is verified by the observation of expulsion of secretions in which spermatozoa are embedded. Razi *et al.* (2010) observed a high distribution of mononuclear cells intraepithelially in the vas deferens; but they did not describe its possible role.

The presence of glandular cells forming valleys or intercalated in the columnar pseudostratified epithelium of vas deferens together with the observation of a simple coiled tubular gland suggest that V2 count on high secretory activity and possibly is, along with V1, the section where most of the maturation features occur. This assumption is made considering that most spermatozoa contained in distal vas deferens have similar functionality (Ahammad *et al.* 2011*a*, 2011*b*) and chromatin compaction as the ejaculated spermatozoa. Moreover, considering that resorption of spermatozoa has been observed in the epididymis and vas deferens (Tingari 1971), further studies should be needed to investigate the role of these secretions on spermathophagy.

It has been suggested that exosomes are present in avian semen keeping continuous exchanges with spermatozoa performing a modulatory effect on the post gonadic sperm maturation (Santiago-Moreno and Blesbois 2020; Cordeiro *et al.* 2021). Because spermatozoa interacting with the secretions of clear basal round cells were observed, then exosomes from this area could be involved in the delivery of enzymes implied in the chromatin condensation or induction of apoptosis.

In conclusion, we have demonstrated that the transit along the rooster vas deferens results in a high percentage of filiform spermatozoa with compacted chromatin, indicating the importance of a compacted organisation of rooster chromatin before ejaculation, possibly related to motility aspects and penetration capacity, besides ensuring the DNA integrity at the time of fertilisation. The histological study revealed a close apposition of spermatozoa with the epithelium of the vas deferens that might be related to sperm maturation.

References

Ahammad MU, Nishino C, Tatemoto H, Okura N, Kawamoto Y, Okamoto S, Nakada T (2011*a*) Maturational changes in the survivability and fertility of fowl sperm during their passage through the male reproductive tract. *Animal Reproduction Science* **128**, 129–136. doi:10.1016/j.anireprosci.2011.09.010

Ahammad MU, Nishino C, Tatemoto H, Okura N, Kawamoto Y, Okamoto S, Nakada T (2011*b*) Maturational changes in motility, acrosomal proteolytic activity, and penetrability of the inner perivitelline layer of fowl sperm, during their passage through the male genital tract. *Theriogenology* **76**, 1100–1109. doi:10.1016/j.theriogenology.2011.05.017

Ausió J, Soley JT, Burger W, Lewis JD, Barreda D, Cheng KM (1999) The histidine-rich protamine from ostrich and tinamou sperm. A link

between reptile and bird protamines. *Biochemistry* **38**, 180–184. doi:10.1021/bi981621w

Ausió J, González-Romero R, Woodcock CL (2014) Comparative structure of vertebrate sperm chromatin. *Journal of Structural Biology* **188**, 142–155. doi:10.1016/j.jsb.2014.09.004

Banaszewska D, Andraszek K, Biesiada-Drzazga B, Przyborski M (2015) Identification of chromatin proteins in semen of roosters from breeding flocks. *European Poultry Science* **79**, 1–10. doi:10.1399/eps.2015.101

Bauer M, Leigh C, Peirce E, Breed WG (2005) Comparative study of sperm chromatin condensation in the excurrent ducts of the laboratory mouse *Mus musculus* and spinifex hopping mouse *Notomys alexis*. *Reproduction, Fertility and Development* **17**, 611–616. doi:10.1071/RD05027

Bedford JM, Calvin HI (1974) The occurrence and possible functional significance of -S-S- crosslinks in sperm heads, with particular reference to eutherian mammals. *Journal of Experimental Zoology* **188**, 137–155. doi:10.1002/jez.1401880203

Bianchi F, Rousseaux-Prevost R, Sautiere P, Rousseaux J (1992) P2 protamines from human sperm are zinc -finger proteins with one Cys2His2 motif. *Biochemical and Biophysical Research Communications* **182**, 540–547. doi:10.1016/0006-291X(92)91766-J

Bianchi PG, Manicardi GC, Bizzaro D, Bianchi U, Sakkas D (1993) Effect of deoxyribonucleic acid protamination on fluorochrome staining and in situ nick-translation of murine and human mature spermatozoa. *Biology of Reproduction* **49**, 1083–1088. doi:10.1095/biolreprod49.5.1083

Bizzaro D, Manicardi GC, Bianchi PG, Bianchi U, Mariethoz E, Sakkas D (1998) In-situ competition between protamine and fluorochromes for sperm DNA. *Molecular Human Reproduction* **4**, 127–132. doi:10.1093/molehr/4.2.127

Burrows WH, Quinn JP (1937) The collection of spermatozoa from the domestic fowl and turkey. *Poultry Science* **16**, 19–24. doi:10.3382/ps.0160019

Campo JL (1998) Conservation and genetical study of Spanish chicken breeds. In 'Proceedings of the 6th world congress of genetics applied to livestock production, Armidale, 11–16 January 1998'. pp. 155–158. (Univiversity of New England: Armidale)

Chiba A, Nakamura M, Morimoto G (2011) Spermiophagy in the male reproductive tract of some passerine birds. *Zoological Science* **28**, 689–693. doi:10.2108/zsj.28.689

Chiva M, Kasinsky HF, Subirana JA (1987) Characterization of protamines from four avian species. *FEBS Letters* **215**, 237–240. doi:10.1016/0014-5793(87)80153-9

Cordeiro L, Lin H-LH, Carvalho AV, Grasseau I, Uzbekov R, Blesbois E (2021) First insights on seminal extracellular vesicles in chickens of contrasted fertility. *Reproduction* **161**, 489–498. doi:10.1530/REP-20-0462-20-0462

Cornwall GA, Horsten HHV (2007) Sperm maturation in the epididymis. In 'The genetics of male infertility'. (Ed. DT Carrell) pp. 211–231. (Humana Press Inc.: Totowa) doi:10.1007/978-1-59745-176-5_13

Cortés-Gutiérrez EI, Dávila-Rodríguez MI, López-Fernández C, Fernández JL, Gosálvez J (2007) Evaluación del daño en el DNA espermático. *Actas Urológicas Españolas* **31**, 120–131. doi:10.1016/S0210-4806(07)73609-4

Czernik M, Iuso D, Toschi P, Khochbin S, Loi P (2016) Remodeling somatic nuclei via exogenous expression of protamine 1 to create spermatid-like structures for somatic nuclear transfer. *Nature Protocols* **11**, 2170–2188. doi:10.1038/nprot.2016.130

Esponda P, Bedford JM (1985) Surface of the rooster spermatozoon changes in passing through the Wolffian duct. *Journal of Experimental Zoology* **234**, 441–449. doi:10.1002/jez.1402340311

Etches RJ (1996) The male. In'Reproduction in poultry'. (Ed. RJ Etches) pp. 208–223. (CAB International: Oxford)

Fernández-Gonzalez R, Moreira PN, Pérez-Crespo M, Sánchez-Martín M, Ramirez MA, Pericuesta E, Bilbao A, Bermejo-Alvarez P, Hourcade JdD, De Fonseca FR, Gutiérrez-Adán A (2008) Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. *Biology of Reproduction* **78**, 761–772. doi:10.1095/biolreprod.107.065623

Fowler J, Cohen L, Jarvis P (2009) More than two samples-the Kruskal-Wallis test. In 'Pratical statistics for field biology'. (Ed. Wiley) pp. 203–205. (John Wiley and Sons: Chichester)

Gatewood JM, Schroth GP, Schmid CW, Bradbury EM (1990) Zinc-induced secondary structure transitions in human sperm protamines. *Journal of Biological Chemistry* **265**, 20667–20672. doi:10.1016/S0021-9258(17)30555-0

González-Santos JA, Ávalos-Rodríguez A, Martínez-García JA, Rosales-Torres AM, Herrera-Barragán JA (2019) Sperm morphophysiology in different sections of the rooster reproductive tract. *International Journal of Morphology* **37**, 861–866. doi:10.4067/S0717-95022019000300861

Howarth B Jr. (1983) Fertilizing ability of cock spermatozoa from the testis epididymis and vas deferens following intramagnal insemination. *Biology of Reproduction* **28**, 586–590. doi:10.1095/biolreprod28.3.586

Jacobsen CN, Jakobsen M (1999) Flow citometry. In 'Encyclopedia of food microbiology'. (Ed. RK Robinson) pp. 826–834. (Elsevier: Oxford) doi:10.1006/rwfm.1999.0665

Kazerooni T, Asadi N, Jadid L, Kazerooni M, Ghanadi A, Ghaffarpasand F, Kazerooni Y, Zolghadr J (2009) Evaluation of sperm's chromatin quality with acridine orange test, chromomycin A3 and aniline blue staining in couples with unexplained recurrent abortion. *Journal of Assisted Reproduction and Genetics* **26**, 591–596. doi:10.1007/s10815-009-9361-3

Lake PE, Ravie O (1984) An exploration of Cryoprotective compounds for fowl spermatozoa. *British Poultry Science* **25**, 145–150. doi:10.1080/13632758408454852

Louie AJ, Dixon GH (1972) Kinetics of enzymatic modification of the protamines and a proposal for their binding to chromatin. *Journal of Biological Chemistry* **247**, 7962–7968. doi:10.1016/S0021-9258(20)81796-7

Malo AF, Gomendio M, Garde J, Lang-Lenton B, Soler AJ, Roldan ERS (2006) Sperm design and sperm function. *Biology Letters* **2**, 246–249. doi:10.1098/rsbl.2006.0449

Mardani M, Vaez A, Razavi S (2014) Effect of saffron on rat sperm chromatin integrity. *Iranian Journal of Reproductive Medicine* **12**, 343–350.

Mezquita C, Teng CS (1977) Studies on sex-organ development. Changes in nuclear and chromatin composition and genomic activity during spermatogenesis in the maturing rooster testis. *Biochemical Journal* **164**, 99–111. doi:10.1042/bj1640099

Morris SA, Howarth B Jr., Crim JW, de Cordoba SR, Esponda P, Bedford JM (1987) Specificity of sperm-binding Wolffian duct proteins in the rooster and their persistence on spermatozoa in the female host glands. *Journal of Experimental Zoology* **242**, 189–198. doi:10.1002/jez.1402420210

Munro SS (1935) Motility and fertilizing capacities of fowl sperm in the excretory ducts. *Proceedings of the Society for Experimental Biology and Medicine* **33**, 255–257. doi:10.3181/00379727-33-8331P

Munro SS (1938) Functional changes in fowl sperm during their passage through the excurrent ducts of the male. *Journal of Experimental Zoology* **79**, 71–92. doi:10.1002/jez.1400790106

Nicander L, Hellström B (1967) Increased thickness of the inner mitochondrial membrane during sperm maturation in the domestic rooster. *Experimental Cell Research* **48**, 622–625. doi:10.1016/0014-4827(67)90329-1

Oliva R, Dixon GH (1991) Vertebrate protamine genes and the histone-to-protamine replacement reaction. In 'Progress in nucleic acid research and molecular biology. Vol. 40'. (Eds WE Cohn, K Moldave) pp. 25–94. (Academic Press) doi:10.1016/s0079-6603(08)60839-9

Oliva R, Mezquita C (1986) Marked differences in the ability of distinct protamines to disassemble nucleosomal core particles *in vitro*. *Biochemistry* **25**, 6508–6511. doi:10.1021/bi00369a025

Razi M, Hasanzadeh S, Najafi G, Feizi S, Moshtaghi M, Janbaz H, Amin M (2010) Histological and anatomical study of the White Rooster: testis, epididymis and ductus deferens. *Iranian Journal of Veterinary Medicine* **4**, 229–236. doi:10.22059/ijvm.2010.22097

Rhim JA, Connor W, Dixon GH, Harendza CJ, Evenson DP, Palmiter RD, Brinster RL (1995) Expression of an avian protamine in transgenic mice disrupts chromatin structure in spermatozoa. *Biology of Reproduction* **52**, 20–32. doi:10.1095/biolreprod52.1.20

Robaire B, Hinton BT (2015) Chapter 17 - The epididymis. In 'Knobil and Neill's Physiology of reproduction'. (Eds TM Plant, AJ Zeleznik) pp. 691–771. (Academic Press) doi:10.1016/b978-0-12-397175-3.00017-x

Santiago-Moreno J, Blesbois E (2020) Functional aspects of seminal plasma in bird reproduction. *International Journal of Molecular Sciences* **21**, 5664. doi:10.3390/ijms21165664

Santiago-Moreno J, Castaño C, Toledano-Díaz A, Coloma MA, López-Sebastián A, Prieto MT, Campo JL (2011) Semen cryopreservation for the creation of a Spanish poultry breeds cryobank: optimization of freezing rate and equilibration time. *Poultry Science* **90**, 2047–2053. doi:10.3382/ps.2011-01355

Santiago-Moreno J, Castaño C, Toledano-Díaz A, Coloma MA, López-Sebastián A, Prieto MT, Campo JL (2012) Influence of season on the freezability of free-range poultry semen. *Reproduction in Domestic Animals* **47**, 578–583. doi:10.1111/j.1439-0531.2011.01921.x

Siegel S, Castellan NJ (1995) Análisis de la Varianza unifactorial por rangos, de Kruskal-Wallis. In 'Estadística no paramétrica: aplicada a las ciencias de la conducta'. pp. 240–250. (Trillas: Mexico, DF)

Simões R, Feitosa WB, Mendes CM, Marques MG, Nicacio AC, de Barros FRO, Visintin JA, Assumpção M (2009) Use of chromomycin A3 staining in bovine sperm cells for detection of protamine deficiency. *Biotechnic & Histochemistry* **84**, 79–83. doi:10.1080/10520290902843595

Słowińska M, Paukszto Ł, Paweł Jastrzębski J, Bukowska J, Kozłowski K, Jankowski J, Ciereszko A (2020) Transcriptome analysis of turkey (*Meleagris gallopavo*) reproductive tract revealed key pathways regulating spermatogenesis and post-testicular sperm maturation. *Poultry Science* **99**, 6094–6118. doi:10.1016/j.psj.2020.07.031

Tingari MD (1971) On the structure of the epididymal region and ductus deferens of the domestic fowl (*Gallus domesticus*). *Journal of Anatomy* **109**, 423–435.

Tingari MD, Lake PE (1972) Ultrastructural evidence for resorption of spermatozoa and testicular fluid in the excurrent ducts of the testis of the domestic fowl, *Gallus domesticus*. *Journal of Reproduction and Fertility* **31**, 373–381. doi:10.1530/jrf.0.0310373

Toshimori K (2003) Biology of spermatozoa maturation: an overview with an introduction to this issue. *Microscopy Research and Technique* **61**, 1–6. doi:10.1002/jemt.10311

Villaverde-Morcillo S, Esteso MC, Castaño C, Toledano Díaz A, López-Sebastián A, Campo JL, Santiago-Moreno J (2015) Influence of staining method on the values of avian sperm head morphometric variables. *Reproduction in Domestic Animals* **50**, 750–755. doi:10.1111/rda.12574

Villaverde-Morcillo S, Esteso MC, Castaño C, Santiago-Moreno J (2016) Influence of post-mortem sperm recovery method and extender on unstored and refrigerated rooster sperm variables. *Reproduction in Domestic Animals* **51**, 40–46. doi:10.1111/rda.12643

Wishart GJ, Horrocks AJ (2000) Fertilization in birds. In 'Fertilization in protozoa and metazoan animals'. (Eds JJ Tarín, A Cano) pp. 193–222. (Springer: Berlin) doi:10.1007/978-3-642-58301-8_5

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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