PHYSIOLOGY AND REPRODUCTION

Expression and secretion of albumin in male turkey (*Meleagris gallopavo*) reproductive tract in relation to yellow semen syndrome[1]

M. Słowińska,[*,2] A. Hejmej,[†] J. Bukowska,[‡] E. Liszewska,[*] B. Bilińska,[†] P. Hliwa,[§] K. Kozłowski,[#] J. Jankowski,[#] and A. Ciereszko[*]

[*]*Department of Gamete and Embryo Biology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences in Olsztyn, Olsztyn 10-748, Poland;* [†]*Department of Endocrinology, Institute of Zoology and Biomedical Research, Jagiellonian University, Krakow 30-387, Poland;* [‡]*Department of Biological Function of Food, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences in Olsztyn, Olsztyn 10-748, Poland;* [§]*Department of Ichthyology, Faculty of Environmental Sciences, University of Warmia and Mazury in Olsztyn, Olsztyn 10-719, Poland; and* [#]*Department of Poultry Science, Faculty of Animal Bioengineering, University of Warmia and Mazury in Olsztyn, Olsztyn 10-719, Poland*

ABSTRACT Yellow semen syndrome (YSS) is the most widely recognized problem among male turkeys. Yellow semen is of low quality and, when used for insemination, results in reduction of fertility and hatchability. Elevated level of serum albumin-like protein accession no. XP˙003205725 is a characteristic feature of yellow seminal plasma suggesting albumin role in YSS pathology. However, knowledge regarding the expression of albumin in the reproductive tract in relation to YSS is very limited. The aim of this study was to identify albumin secretion and localization sites in the turkey reproductive tract in relation to YSS. Reproductive tract tissues and liver originating from turkeys producing white semen (WS) and YSS were used for analysis of albumin mRNA expression and its localization using immunohistochemistry. Moreover, albumin abundance in tissues, blood and seminal plasma was analyzed using two dimensional electrophoresis and western blot analysis. Albumin mRNA expression was found in all parts of the reproductive tract. Apart from the liver, the highest expression of albumin was found in the ductus deferens in YSS turkeys. The testicular spermatids, Leydig, and myoid cells and the epithelium of the epididymis and ductus deferens were the main secretion sites of albumin in the reproductive tract in turkeys. Higher albumin abundance was found in the reproductive tract and seminal plasma of YSS toms compared to WS toms. Our results demonstrated that germ cells from spermatocytes to spermatids, Leydig cells, and myoid cells synthesized and secreted albumin in turkey testis, and epithelial cells are the main secretion sites in epididymis and ductus deferens. Ductus deferens secretion of albumin seems to be mostly responsible for YSS. Over-secretion by the ductus deferens may be the main origin of albumin abundance in YSS semen. Knowledge regarding disturbances of albumin secretion in relation to YSS may be useful for future work on studies related to better understanding the molecular basis of YSS.

Key words: birds, sperm, reproductive system, seminal plasma proteins

2019 Poultry Science 98:1872–1882
http://dx.doi.org/10.3382/ps/pey490

INTRODUCTION

Yellow semen syndrome (**YSS**) is the most widely recognized problem among male turkeys (Sexton, 1976). Turkeys with YSS produce semen of yellow color that is associated with low semen quality such as: sperm morphology and biochemical parameters of seminal plasma, and consequently leads to reduction of fertility and hatchability when used for artificial insemination (Thurston et al., 1982, 1992; Hess and Thurston, 1984a; Thurston and Korn, 1997). The etiology of YSS in turkeys is mostly unknown. However, suboptimal photoperiods, as well as genetic propensity for YSS and inbreeding for improvement body conformation and egg production may contribute to pathology of YSS (Thurston and Korn, 1997). Although, semen volume and sperm numbers of YSS males do not differ from white semen (**WS**) males, yellow turkey semen contains abnormal spermatozoa and spermatids, and often increased numbers of spermiophages and bacteria (Thurston and Korn, 1997). The pathology of YSS has also been identified in the reproductive tract, especially in the ductuli efferentes where epithelial cells were

Received July 2, 2018.
Accepted September 20, 2018.
[1]This work was funded by the Institute of Animal Reproduction and Food Research of Polish Academy of Sciences.
[2]Corresponding author: m.slowinska@pan.olsztyn.pl

hypertrophied and filled with lipid-like droplets (Hess et al., 1982; Thurston and Korn, 1997).

Studies on biochemical characteristics of seminal plasma appear to have been useful in revealing differences between YSS and WS. In particular, an elevated protein concentration has been shown to be a characteristic feature of YSS (Thurston et al., 1982; Hess and Thurston, 1984b) and was used to screen for YSS in commercial flocks (Hess and Thurston, 1984a). Moreover, YSS seminal plasma is characterized by increased activities of aspartate aminotransaminase, acid phosphatase, and superoxide dismutase and increased levels of cholesterol (Hess and Thurston, 1984b; Słowińska et al., 2011). So far, the detailed mechanism leading to over-secretion of these proteins is unknown.

Application of difference in-gel electrophoresis for comparison of white and yellow seminal plasma proteome has enabled detection of potential semen protein markers for YSS (Słowińska et al., 2015a). Elevated levels of albumin, transthyretin, hemopexin, immunoglobulin, and pantetheinase-like protein were found in yellow seminal plasma (Słowińska et al., 2015a). Among those proteins, albumin was found to differentiate mostly white and yellow seminal plasma. Higher concentrations of albumin in yellow seminal plasma were also postulated by Thurston et al. (1982). Further studies indicated that similarly to chicken (Labas et al., 2015) albumin is one the most dominant proteins in turkey seminal plasma (Słowińska et al., 2017).

Albumin is the major protein of biological fluids, including semen. Its origin in the male reproductive system can be related to blood but testicular, epididymal, and prostatic origins are also suggested (Elzanaty et al., 2007). Seminal plasma albumin can affect sperm plasma membrane lipid composition through lipid exchange or hydrolysis (Go and Wolf 1985). Mammalian albumin is involved in the transport of molecules to the sperm membrane during epididymal maturation, in the movement of proteins during the acrosomal reaction, or in membrane remodeling during sperm–oocyte membrane fusion (Arroteia et al., 2014). Albumin has also been found intracellularly and a high glycoprotein complex containing albumin is present in the sperm acrosome (Arroteia et al., 2014). All available information concerning the role of albumin in male reproductive system concerns mammals. To our knowledge such information is not available for birds.

Despite the higher abundance of albumin in yellow seminal plasma (Słowińska et al., 2015a), which suggests its role in YSS, knowledge regarding albumin secretion and localization in the turkey reproductive tract in relation to YSS is very limited. In order to understand the molecular basis of YSS it is very important to determine whether pathology related to over-secretion of albumin involves only the reproductive tract or whether other organs, such as liver, are involved. It is still controversial if albumin in turkey seminal plasma originates from secretion in the reproductive tract and/or from blood, suggested previously by Thurston et al. (1982). Identification of the main sites of altered albumin secretion in the reproductive tract is prerequisite for a better understanding of the mechanism involved in YSS pathology. This knowledge may be useful for future work on studies related to better understanding the molecular basis of YSS.

MATERIALS AND METHODS

Birds, Housing, and Tissue Collection

Turkeys (British United Turkeys Big 6, Grelier, Saint-Laurent-de-la-Plaine, France) were maintained under standard husbandry conditions at the Turkey Testing Farm of the Department of Poultry Science (University of Warmia and Mazury in Olsztyn, Poland). Feed and water were provided ad libitum. Males were photostimulated at 26 wk of age (14 h light to 10 h darkness) and produced semen by 30 wk of age. Individual semen samples were collected at 1-wk intervals by abdominal massage (Burrows and Quinn, 1937). Semen was centrifuged twice for 10 min at 7,950 × g at 4°C (Thurston et al., 1993; Holsberger et al., 2002). We considered the supernatant as seminal plasma, which was stored at –26°C.

The liver and the male reproductive tract tissue samples were obtained from twelve 38-wk-old turkeys killed in a local slaughterhouse, six of them produced WS and six were characterized by YSS. For quantitative RT-PCR, the tissues were immediately frozen in liquid nitrogen, and for immunohistochemical study, the tissues were fixed in Bouin's fluid. The blood was centrifuged for 10 min at 1,605 × g. The resulting blood plasma was stored at –26°C.

White and Yellow Semen Classification

Thurston et al.'s (1982) criteria were used for white and yellow semen classification. Semen with a white color and a low seminal plasma protein concentration (≤20 mg/mL) was classified as normal white. Semen with a yellow color and a high seminal plasma protein concentration (>20 mg/mL) was classified as abnormal yellow. The protein concentration was measured using the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Although the measurement of the total protein concentration in seminal plasma may not be related to albumin concentration it has been successfully used for the discrimination of white and yellow semen (Thurston et al., 1982). Similar criteria were previously used by Słowińska et al., 2015a and Pardyak et al., 2018.

Semen Quality Analysis

The percentage of motile spermatozoa in six samples of white and six samples of yellow semen was analyzed using a computer-aided sperm analysis with Hobson Sperm Tracker (Hobson Vision Ltd., Baslow, UK) and MicroCell 2-chamber (20-μm depth

of the chamber) slides (Conception Technologies, San Diego, CA) according to Słowińska et al. (2018). The program settings for the image analysis at the ×10 objective magnification were as follows: search radius = 11.63 μm; predict = off; video = pal; aspect = 1.49; refresh time = 1 s; threshold +15/−90; filter weightings 1 = 2, 2 = 2, 3 = 2, 4 = 2; and image capture rate = 50 Hz. Analyses were done in duplicate; 100 sperm tracks were recorded from each side of the slide, for a total of 200 tracks/sample. Semen samples were incubated for 5 min in motility buffer (50 mM Tris buffer, pH 7.4, 120 mM NaCl, 10 mM glucose, and 2 mM $CaCl_2$) at 39°C. The motility buffer composition used in the present study was previously adapted for CASA analysis of turkey spermatozoa by King et al. (2000) and applied in our studies on turkey semen storage (Kotłowska et al., 2007; Słowińska et al., 2012, 2013, 2018). The study of Lemoine et al. (2008) showed no induction of acrosome reaction of chicken semen in NaCl-TES buffer (applied in present study) in the presence of Ca^{2+} and bovine serum albumin (**BSA**) if buffer was not supplemented with the inner perivitelline layer. Semen was diluted 300 to 500 times to obtain 30 to 50 sperm on the screen at the start of motility. The motility buffer was supplemented with 0.5% BSA to prevent adherence of spermatozoa to the glass slides.

Sperm concentration and viability were determined in six samples of white and six samples of yellow semen by flow cytometry using a Muse Cell Analyzer (EMD Millipore, Billerica, USA) and Muse Count and Viability Reagent (EMD Millipore) according to Słowińska et al. (2018). In this technique, a membrane-permeant DNA-staining dye stains all cells with nuclei and is then used to discriminate these nucleated cells from debris and non-nucleated cells. A DNA-binding Muse dye based on 7-aminoactinomycin D stains cells that have lost their membrane integrity, allowing the dye to stain the nucleus of dead and dying cells. This parameter was displayed as viability and was used to discriminate viable (live cells that do not stain) from non-viable (dead or dying cells that stain). Semen was diluted 3,000 times in phosphate-buffered saline to obtain concentrations in the range of 1×10^5 to 1×10^7 sperm/mL. Subsequently, 20 μL of each semen suspension was mixed with 380 μL of Muse Count and Viability Reagent and incubated for 5 min at room temperature.

RNA Isolation and Real-Time PCR

RNA isolation and real-time PCR were performed according to the methods described previously (Słowińska et al., 2015b). Briefly, total RNA was extracted from the samples using a Total RNA Mini kit (A&A Biotechnology) according to the manufacturer's instructions. The RNA concentration and quality were determined spectrophotometrically using a ND-1000 spectrophotometer (NanoDrop Technologies) and agarose gel electrophoresis. Genomic DNA was removed from RNA samples using DNase I digestion (Invitrogen Life Technologies, Inc.). Total RNA (1,000 ng) was reverse transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, MA, USA) according to the manufacturer's protocol. Real-time PCR for albumin (*ALB*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; chosen as a housekeeping gene) mRNA levels was performed using the following primers: *ALB:* 5′-CACTCCTGGAAAAGTGCTGC-3′ (forward), 5′-AGCGGATCAGGATGGACTTG-3′ (reverse); *GAPDH:* 5′-GATCCCTTCATCGACCTGAA-3′ (forward), 5′-CATCTGCCCACTTGATGTTG-3′ (reverse). The total reaction volume for PCR contained 1 μL of complementary DNA (50 ng), 0.4 μL of (200 nM) forward and reverse primers each, 3 μL of nuclease-free water, 5 μL of KAPA SYBR Fast qPCR master mix, and 0.2 μL of ROX Low reference dye (KAPA Biosystems, Wilmington, MA, USA) in a final volume of 10 μL. The thermal cycling conditions were as follows: 95°C for 3 min (initial denaturing), followed by 40 repeated cycles of 95°C for 15 s (denaturing), and 59°C for 30 s (annealing). To ensure proper amplification of the single-product after each PCR reaction, melting curves were obtained by stepwise increases in temperature. Data obtained in real-time were normalized on the basis of *GAPDH* mRNA content and analyzed using the Zhao and Fernald method (Zhao and Fernald, 2005).

Immunohistochemical Detection of Seminal Plasma Albumin in Turkey Reproductive Tissues and Liver

Fragments of testis, epididymis, ductus deferens, and liver obtained from turkeys (n = 6) with normal WS and turkeys (n = 6) with abnormal YSS were fixed in Bouin's fluid (saturated picric acid, formaldehyde, and glacial acetic acid at 15:5:1 proportion) for 24 h, dehydrated in an increasing gradient of ethanol and embedded in paraffin. Epididymis was identified based on previous studies (Hess et al., 1976; Hess and Thurston, 1977; Hess et al., 1982) and our own experience as described Kotłowska et al. (2005). All sections (5-μm thickness) were mounted on slides coated with 3-aminopropyltriethoxysilane (APES; Sigma-Aldrich), deparaffinized in xylene, rehydrated gradually through a series of ethanol dilutions, and washed in water (Kotula-Balak et al., 2008). The procedures used for immunohistochemistry were similar to those described recently by Słowińska et al. (2014, 2015b). To achieve antigen retrieval, the slices were immersed in citrate buffer (10 mM, pH 6.0) and heated in a microwave oven (8 min, 750 W). Endogenous peroxidase activity was blocked by incubation in a solution of hydrogen peroxide (0.3%, v/v) in TBS (Tris buffered saline, 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.6), while non-specific binding was prevented by using

normal goat serum (5%, v/v). Thereafter, sections were incubated overnight at 4°C in a humidified chamber in the presence of antibodies against albumin (dilution 1:500) produced previously in our laboratory (Słowińska et al., 2015a). Subsequently, sections were incubated with biotinylated secondary antibody, goat anti-rabbit IgG (1:400; Vector Lab., Burlingame CA, USA), respectively, for 60 min. After each step in these procedures, sections were carefully rinsed with TBS; the antibodies were also diluted in TBS buffer. The staining was developed using avidin-biotinylated horseradish peroxidase complex (ABC/HRP; 1:100; VECTASTAIN Elite ABC Reagent, Vector Lab.) for 30 min followed by 0.05% 3,3'-diaminobenzidine tetrachloride (DAB; Sigma-Aldrich) in TBS containing 0.01% H_2O_2 and 0.07% imidazole for 6 min. Thereafter, sections were washed and counterstained with Mayer's hematoxylin, dehydrated, and mounted using DPX mounting media (Sigma-Aldrich). All slides were processed immunohistochemically at the same time with the same treatment so that staining intensity of the different sections could be compared (Hejmej et al., 2005). The cells were considered immunopositive if brown reaction product was present and appeared as a signal in reproductive tissue cells; the cells without any specific immunostaining were considered immunonegative (Dietrich et al. 2014). Negative controls included sections incubated with pre-immune goat serum instead of primary antibody. All immunohistochemical experiments were repeated three times. The sections were examined using a Leica DMR microscope (Leica, Microsystems GmbH Wetzlar, Wetzlar, Germany).

Two-Dimensional Gel Electrophoresis and Western Blot

Tissues frozen in liquid nitrogen were thawed on ice and 100 μg of tissues were sonicated using VC-13 PB (Sonics, USA), set at 35% relative output, for 30 s in 200 μL extraction buffer containing 7 M urea, 2 M thiourea, 4% CHAPS (wt/vol), 40 mM DTT, and 2% IPG buffer. The tissue extracts were then centrifuged at 12,800 × g for 20 min at 4°C and 100 μg protein (in replicates) was precipitated using the 2-D Clean-up Kit (GE Healthcare, Uppsala, Sweden). The pellet was re-suspended in rehydration buffer containing 7 M urea, 2 M thiourea, 2% CHAPS (wt/vol), 18 mM DTT, 2% IPG buffer, and a trace of bromophenol. The protein concentration was measured by the method of Bradford (1976) using a Coomassie Plus Kit (Thermo Scientific, Waltham, MA, USA) with bovine serum albumin as standard. The samples (100 μg protein) were then loaded onto immobiline DryStrip gels (IPG) strips (7 cm, pH 4–7 NL; GE Healthcare) with passive rehydration (18 h). Isoelectric focusing was performed with an IPGphor isoelectric focusing unit (GE Healthcare) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was run using the SE 250 vertical Mighty Small electrophoresis system (GE Healthcare), as described by Słowińska et al. (2015a). Seminal plasma and blood samples were prepared for two-dimensional gel electrophoresis according to Słowińska et al. (2015a).

A western blot was performed as described by Słowińska et al. (2015a). Four samples each of white and yellow reproductive tract tissues, seminal plasma, liver, and blood were transferred to nitrocellulose membranes. Polyclonal antibodies against the albumin were diluted with TBS-T (0.05 M Tris-HCl, 0.15 M NaCl, and 0.1% Tween 20, pH 7.6) at a ratio of 1:10,000. Blots were scanned using the VersaDoc MP 4000 system (Bio-Rad) and albumin spots were quantified by area (mm^2) using Quantity One Analysis Software (version 4.6.9, Bio-Rad).

Statistics

The results of mRNA albumin expression and albumin area were analyzed using two-way analysis of variance. Significant differences between mean values were determined by least significant difference tests. The differences were considered significant at $P \leq 0.05$. Statistical (Version 13.1, 2016 StatSoft Inc., Tulsa, OK) software was used for statistical calculations. The differences in semen quality parameters between white and yellow semen were analyzed by the Student's t-test using the statistical software program GraphPad5 (GraphPad PRISM version 5.0, GraphPad Software Inc., San Diego, CA). Data were presented as mean ± SEM. Correlation was calculated using the Spearman correlation test.

RESULTS

White and Yellow Semen Quality Parameters

Sperm concentration ($P = 0.35$) and sperm motility ($P = 0.15$) did not differ between white and yellow semen. However, yellow semen was characterized by slightly and significantly lower sperm viability ($P = 0.026$). The lack of differences between white and yellow sperm motility could possibly result from higher variability data for sperm motility compared to viability. The protein concentration clearly distinguished white and yellow seminal plasma and was 4.5 times higher in YSS semen than that of WS (Table 1). There was no correlation between sperm quality parameters and albumin abundance measurement within reproductive tract (data not shown).

Expression of ALB mRNA in the Male Reproductive Tract

Albumin mRNA transcripts were detected in the turkey reproductive tract and liver. Within the reproductive tract, the highest levels of ALB mRNA

Table 1. Quality parameters of white and yellow turkey semen.

Parameters	White	Yellow
Sperm concentration ($\times 10^9$/ml)	9.49 ± 1.5[a]	8.67 ± 1.11[a]
Sperm viability (%)	97.85 ± 0.28[a]	97.03 ± 0.76[b]
Motility (%)	74.17 ± 9.55[a]	67.83 ± 3.87[a]
Protein concentration in seminal plasma (mg/ml)	16.96 ± 6.70[a]	77.19 ± 24.01[b]

[a,b]Means with different superscripts are significantly different ($P \leq 0.05$).
Number of samples = 6/group.
Results are reported as means ± SEM.

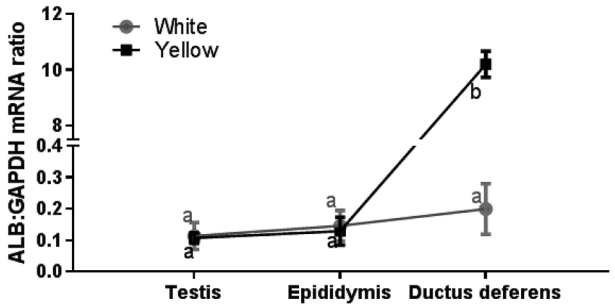

Figure 1. Expression of albumin mRNA in male reproductive tract tissues of WS and YSS turkeys. Data are presented as mean values ± SEM. Different letters indicate statistical significance at $P \leq 0.05$.

transcripts were detected in the ductus deferens of YSS males, but not in males producing WS (Figure 1). Despite significant histological differences, we did not observe any differences in ALB expression in testis and epididymis between WS and YSS males. However, a dramatic difference was found in the ductus deferens, where expression observed in YSS males was 51-fold that of WS males. There was no difference between YSS and WS males in terms of ALB expression in the liver (492.1 ± 16.6 vs 421.2 ± 64.5 for the ALB:GADPH mRNA ratio for white and yellow males, respectively, $P = 0.71$).

Immunohistochemical Detection of Albumin in Turkey Reproductive Tissues and Liver

Immunohistochemical study revealed positive signals of various intensities for albumin in the testis, epididymis, ductus deferens, and liver of WS and YSS turkeys (Figure 2A–H). In the testis of WS turkeys, a positive signal for albumin was localized in germ cells (except for spermatogonia), being of moderate to strong intensity in spermatocytes and round and elongating spermatids (Figure 2A). Leydig cells within the interstitial tissue and myoid cells also exhibited moderate to strong immunoreaction (Figure 2A'). Thorough analysis at higher magnification revealed a linear staining pattern on the plasma membrane of spermatocytes that may suggest membrane localization of albumin (bottom image of Figure 2A). A strong signal was particularly evident in elongating spermatids with localization in the cytoplasmic droplets. In the testis of YSS turkeys, the intensity of immunoreaction for albumin was clearly reduced in spermatocytes (Figure 2B). However, a linear staining pattern remained on their plasma membrane, as shown in the higher magnification image (bottom image of Figure 2B). In some tubules, elongating spermatids displayed either a strong signal for albumin (Figure 2B') as observed in the WS turkeys or a reduced one (Figure 2B). In Leydig cells, a positive signal of moderate intensity was observed, while myoid cells exhibited strong immunostaining for albumin (Figure 2B). Independently of the turkey group, the epithelium around blood vessels was positively stained (not shown). No positive immunoreaction was detected at the base of the epithelium, either in Sertoli cells or spermatogonia (Figure 2A and B, and bottom images of Figure 2A and B).

In the proximal epididymal region in tissue from WS turkeys, a strong signal for albumin was confined to the apical cytoplasm of most epithelial cells (Figure 2C). Concomitantly, in some cells a weak, diffused staining was noticed. In the ductus epididymis, non-ciliated epithelial cells displayed a strong staining for albumin (Figure 2C'). Some epithelial cells projected considerable amounts of positively stained cytoplasm into the lumen as did loose cytoplasmic droplets (Figure 2C'). There was no positive staining in the ciliated epithelial cells, basal cells, and spermatozoa without cytoplasmic droplets (Figure 2C'). In the proximal epididymis of YSS turkeys, the apical cytoplasm of the epithelial cells displayed a strong signal for albumin, while in the rest of the cytoplasm various intensities of signal were observed (Figure 2D). Along the ductus epididymis, delocalization of albumin to the perinuclear region of non-ciliated epithelial cells occurred (Figure 2D'). Moreover, microvilli projected from non-ciliated cells and cell cytoplasmic protrusions were positively stained. In contrast, ciliated and basal epithelial cells and sperm did not exhibit any staining (Figure 2D').

In the pseudostratified columnar epithelium of the ductus deferens from WS turkeys, a strong staining for albumin was observed, mainly within the epithelial cell cytoplasm (Figure 2E). In some cells, a strong diffused staining was observed. Basal cells were weakly stained (Figure 2E). In contrast, in the ductus deferens of YSS turkeys, the intensity of immunoreaction for albumin was very strong only in the apical epithelium (Figure 2F). As shown in Figure 2E' and F', evident cytoplasmic distribution of albumin was observed irrespective of the turkeys groups, although in YSS turkeys the signal intensity was significantly reduced (Figure 2F' vs E'). In the lumen, there were positively stained cell cytoplasmic protrusions, namely loose cytoplasmic droplets (Figure 2E' and F').

In the liver cells of WS and YSS turkeys, the positive signal for albumin was of moderate intensity and localized in several hepatocytes, either in their cytoplasm or their nuclei (Figure 2G and H).

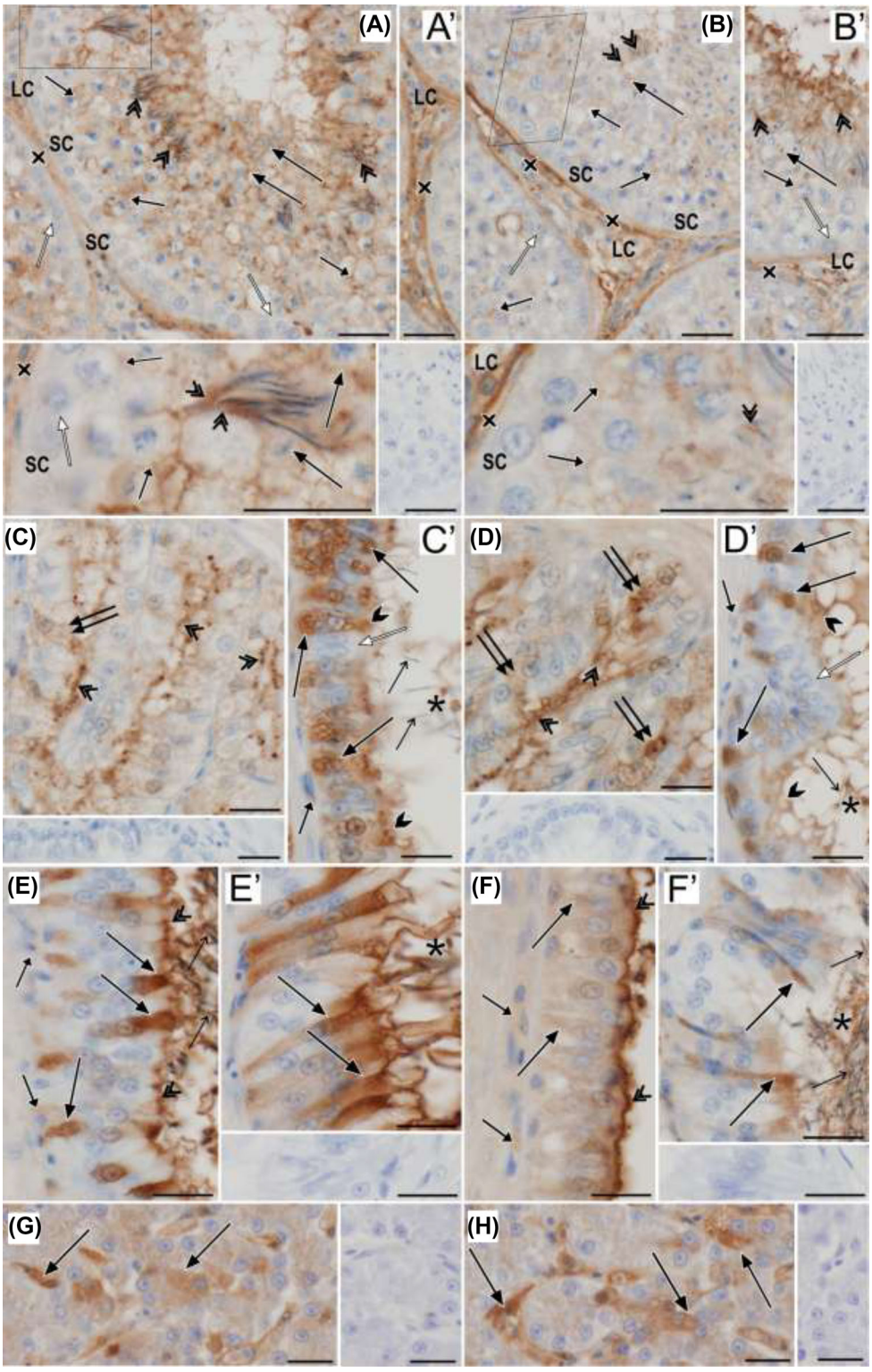

Figure 2. Representative microphotographs showing immunohistochemical localization of albumin in WS turkeys (A, A', C, C', E, E', G) and YSS turkeys (B, B', D, D', F, F', H) in the testis (A, A' B, B'), epididymis (C, C', D, D'), ductus deferens (E, E', F, F'), and liver (G, H). Immunostaining was performed using seminal plasma antibody against albumin, followed by anti-rabbit IgG, and ABC/HRP visualized by DAB. Samples were counterstained with Mayer's haematoxylin. Bars = 10 μm. Frames indicate the location of a higher magnification view. (A, A') Moderate to strong signal for albumin is localized in spermatocytes (short arrows), round spermatids (arrows), and elongated spermatids (double arrowheads). Note strong signal, in Leydig cells within the interstitial tissue (LC) and myoid cells (crosses). No immunopositive reaction is observed in Sertoli cells (SC) and spermatogonia (white arrows). At higher magnification (bottom [boxed image from main panel]), a strong, linear staining pattern is visible on the plasma membrane of spermatocytes (short arrows). Note the strong signal localized in cytoplasmic droplets of elongating spermatids (double arrowheads). (B, B') Weak signal for albumin is localized in spermatocytes (short arrows). Note either weak (B) or strong (B') signal in elongating spermatids (double arrowheads). Leydig cells are moderately stained (LC), while a moderate to strong signal is localized in myoid cells (crosses). No immunopositive reaction is observed in Sertoli cells (SC) and spermatogonia (white arrows).

In the control sections, in which the primary antibody was omitted and replaced with pre-immune serum, no immunopositive signals for albumin were observed.

Albumin Pattern in the Male Reproductive Tract, Seminal Plasma, Liver and Blood

Polyclonal antibodies against turkey seminal plasma ALB recognized albumin proteoforms in the testis, epididymis, ductus deferens, seminal plasma, liver and blood of WS and YSS turkeys (Figure 3A). Albumin spots with greater albumin area were observed in the reproductive tract and seminal plasma of YSS turkeys (Figure 3B). On the other hand, there were no differences between YYS and WS males in terms of albumin area found in liver tissue and blood (Figure 3C).

DISCUSSION

In the present study, we demonstrated the expression of albumin mRNA in the turkey reproductive tract. Apart from the liver, the highest expression of albumin was found in the ductus deferens in turkeys with YSS. Testicular spermatids, Leydig, and myoid cells and epithelium of the epididymis and ductus deferens were the main secretion sites of albumin in the turkey reproductive tract. Polyclonal anti-albumin antibodies cross-reacted with reproductive tissue, seminal plasma, liver, and blood. Higher albumin abundance was found in the reproductive tract and seminal plasma of YSS toms compared to WS toms.

The presence of albumin in turkey seminal plasma has been reported by Thurston et al. (1982) and confirmed by Słowińska et al. (2015a). However, so far the origin of albumin in turkey seminal plasma is controversial. Thurston et al. (1982) suggested that either blood proteins are transported to semen or they may be synthesized and released by reproductive tissue in turkeys. It is possible that both of these origins may be involved. Skinner et al. (1987) estimated that 10% to 20% of transferrin in ram rete testis fluid originates from blood and 80% to 90% from the testis. Our results indicated for the first time that cells in the turkey reproductive tract tize and secrete albumin. Further study should focus on establishing the level of albumin secretion in the reproductive tract and/or derived from blood.

In our study, the secretion of albumin occurred in all parts of the reproductive tract of males producing WS. Similar to birds, mammalian albumin mRNA is transcribed in the testis and all parts of the epididymis (Arroteia et al., 2014). This suggests that albumin is important for function of male reproductive tract system in higher vertebrates. Although expression of albumin mRNA in healthy toms producing WS did not differ within the reproductive tract, a steady increase of albumin, detected after western blot analysis, was visible along the reproductive tract, reaching highest values in the ductus deferens where albumin was primarily secreted by epithelial cells (Figure 1). The secretory and absorptive functions of the epithelium are important for maintenance of the microenvironment, especially in the ductus deferens where sperm maturation and storage take place (Clulow and Jones, 1982; Wishart and Horrocks, 2000). Our results strongly suggest that albumin is important part of this mechanism of sperm protection. This suggestion is supported by immunohistochemical results (see below).

The reproductive tract in YSS turkeys was characterized by the highest amount of albumin, detected after western blot analysis, in comparison to WS males. Similar to the reproductive tract in WS turkeys, the highest secretion was observed in the ductus deferens. Moreover, in YSS ductus deferens, we observed overexpression of albumin mRNA, and mRNA expression correlated with albumin secretion within the reproductive tract ($r = 0.81^{*}$). The altered gene expression in YSS males seems to be limited to the reproductive tract, in particular the ductus deferens. It is suggested that further study should examine the mechanisms of disturbances of expression in the ductus deferens of YSS males.

Using immunohistochemistry, we revealed the presence of the albumin in the testis, epididymis, ductus deferens, and liver of healthy WS turkeys. Our study showed that the expression of albumin in the testis is

Figure 2. (Continued) At higher magnification (bottom [boxed image from main panel]), note the linear pattern which remained on the plasma membrane of spermatocytes (short arrows). (C, C') In the proximal epididymal region (C), a strong signal for albumin is localized in the apical cytoplasm of most of the epithelial cells (double arrowheads). In some cells, a weak, diffused staining is visible (double arrows). Along the ductus epididymis (C'), a strong, positive signal for albumin is localized in the cytoplasm of non-ciliated epithelial cells (arrows) and microvilli projected from non-ciliated cells (arrowheads). Positively stained cell cytoplasmic protrusions, namely loose droplets, are visible in the lumen (asterisk). Ciliated epithelial cells (white arrow), basal cells (short arrow), and spermatozoa without cytoplasmic droplets are immunonegative (open arrows). (D, D') A strong signal for albumin is localized in the apical cytoplasm (double arrowheads) of the proximal epididymal region (D). Various signal intensities (weak, moderate, or strong) are localized in epithelial cells (double arrows). Along the ductus epididymis (D'), delocalization of albumin in the perinuclear region of non-ciliated cells (arrows) is visible and microvilli projecting from non-ciliated cells (arrowheads). Positively stained cell cytoplasmic protrusions are visible in the lumen (asterisk). Ciliated cells (white arrow), basal cells (short arrow), and spermatozoa without cytoplasmic droplets are (open arrows) immunonegative. (E, E') Strong staining for albumin is localized in the apical cytoplasm (double arrowheads) of columnar epithelial cells. In some cells, a strong, diffused staining is visible (arrows). Basal cells (short arrows) are weakly stained. Note the evident cytoplasmic pattern of the stain (E'). Positively stained cell cytoplasmic protrusions are visible in the lumen (asterisk). (F, F') Very strong staining for albumin is localized in the apical cytoplasm (double arrowheads) of columnar epithelial cells, while the remainder of the cytoplasm is weakly stained (arrows). In basal cells (short arrows), a very weak signal is observed. Weak cytoplasmic pattern of the staining is clearly visible (F'). Positively stained cell cytoplasmic protrusions are visible in the lumen (asterisk). (G, H) Moderate signal for albumin is localized in hepatocytes (arrows). No immunopositive staining for albumin is observed in testes, epididymides, ductuli deferentes, and liver tissue when the primary antibody is omitted (inserts in A', B', C, D, E', F', G, and H), respectively.

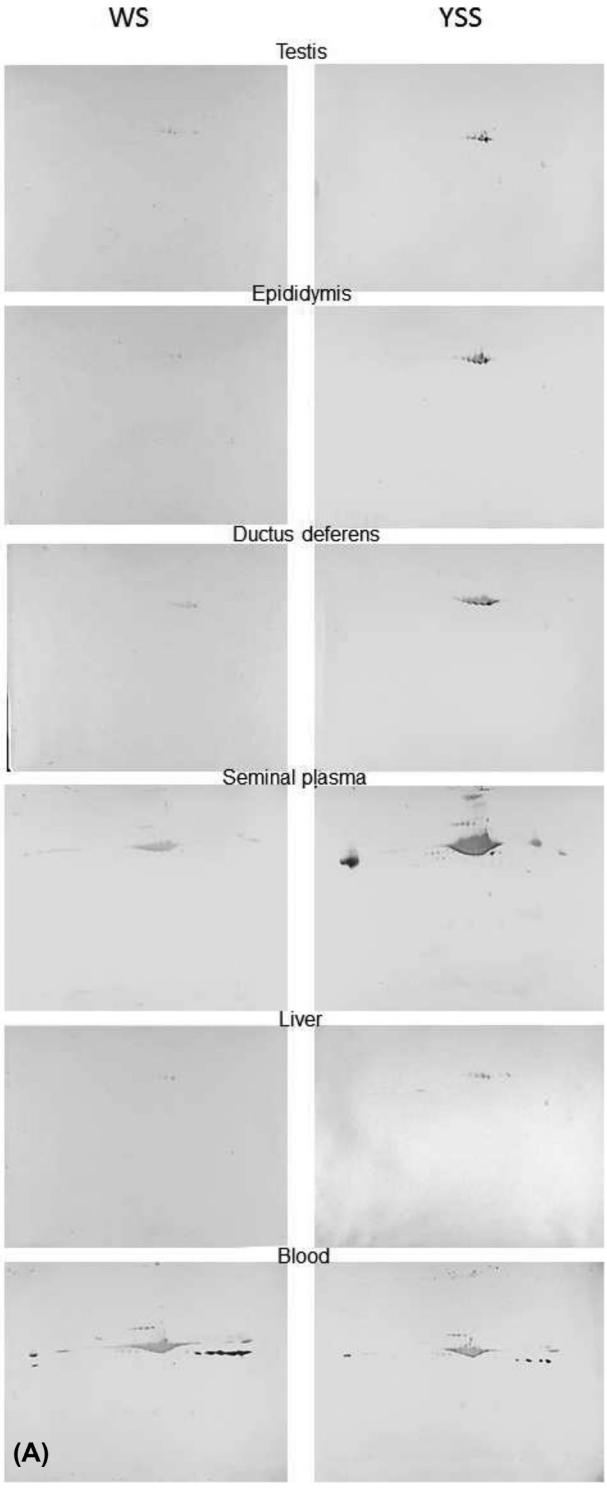

Figure 3. Cross-reactivity between polyclonal antibodies against albumin and turkey reproductive tract tissues, seminal plasma, liver tissue, and blood from males producing WS (white) and YSS (yellow) semen. (A) Proteins were separated by 2DE (two-dimensional gel electrophoresis), transferred to nitrocellulose membrane and visualized using anti-albumin antibodies. Blots were scanned using the VersaDoc MP 4000 system (Bio-Rad, Hercules, CA). (B, C) The areas of albumin spots (mm^2) were quantified using Quantity One Analysis Software (Bio-Rad, Hercules, CA). The data are presented as mean values ± SEM. Different letters indicate statistical significance at $P \leq 0.05$.

linked to specific germ cells, including spermatocytes with membrane albumin localization and round, elongated spermatids in which albumin is localized in the cytoplasmic droplets (Figure 2A). Albumin is known to protect membrane integrity of sperm by elimination of free radicals generated by oxidative stress (Bansal and Bilaspuri, 2011). For this reason it can be suggested that antioxidant properties of albumin may be involved in protection of developing germ cells during spermatogenesis. A similar stage-specific expression of proteins has been previously described for protease inhibitors (Bian et al., 2009; Słowińska et al., 2014, 2015b) and its role in spermatogenesis, particularly for spermatid elongation, was suggested. At present, it is unknown whether albumin is present inside bird spermatozoa. Besides germ cells, the immunohistochemical studies revealed localization of albumin in Leydig and myoid cells of turkey testis. Mainly steroidogenic activity was described for Leydig cells in avian testis (Deviche et al., 2011). This suggests a role of albumin in supporting hormonal production in the reproductive tract of male turkeys. This suggestion is also supported by the increase of dihydrotestosterone binding activity in yellow seminal plasma, characterized by higher abundance of albumin observed by Hess et al. (1984).Those results contradict data concerning the presence of albumin in the mammalian reproductive tract where albumin is localized in Sertoli cells (Elzanaty et al., 2007). The different sites for albumin secretion in mammals and birds suggest differences in albumin function between the two reproductive systems.

In contrast to the testis, in the epididymis and ductus deferens, the albumin was primarily secreted by epithelial cells (Figure 2B and C). Epithelial cells of the epididymis are also known to secrete albumin in mammals (Shaha et al., 1988; Elzanaty et al., 2007). A considerable amount of albumin was projected into the lumen in epithelial cells when cytoplasmic droplets were loose in the epididymis and ductus deferens of WS turkeys. In birds, proteins secreted by epithelial cells of the Wolffian duct (vas deferens) are involved in maturation changes in the sperm membrane (Morris et al., 1987; Esponda, 1991). This indicates that, besides protecting the developing germ cell during spermatogenesis, albumin can be involved in maintaining a suitable microenvironment for sperm maturation and storage, which mainly takes place in the ductus deferens. This suggestion is supported by results of albumin abundance after western blot analysis in healthy turkeys showing the highest secretion of albumin in the ductus deferens (Figure 3B).

It has to be emphasized that there are significant differences between avian and mammalian sperm maturation in epididymis. In contrast to mammals, where maturation is a complex process and involves changes in the lipid and protein composition of the plasmalemma and development of sperm motility, birds have adopted a relatively simple form of spermatozoa maturation or modification along the reproductive tract (Aire, 2007).

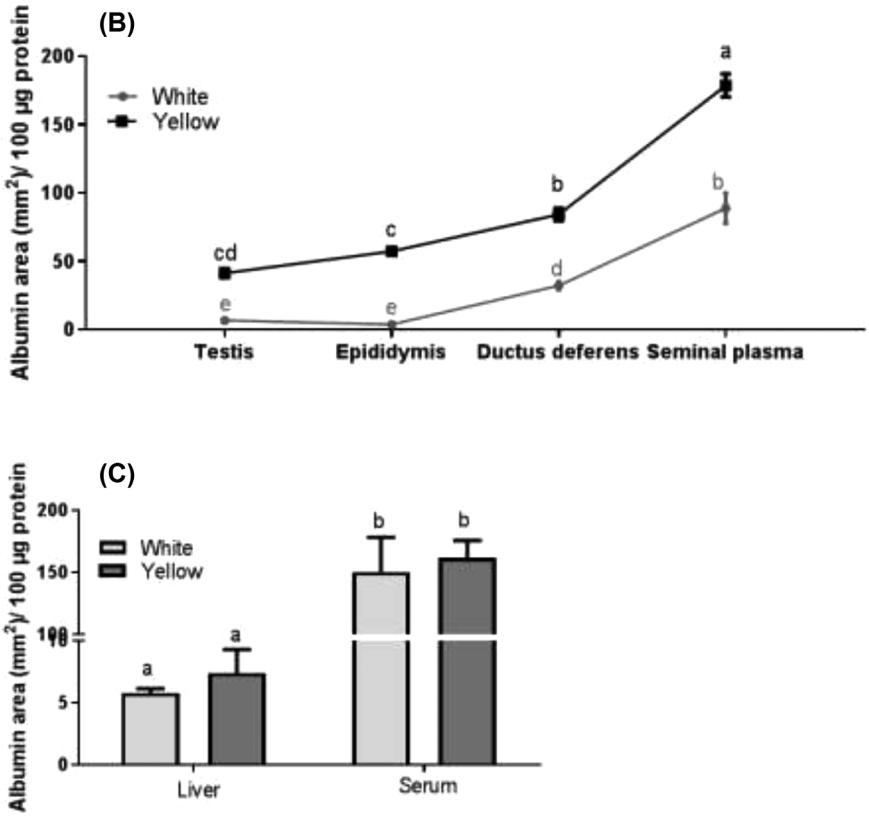

Figure 3. (Continued)

Avian testicular fluid and spermatozoa are produced in large quantities (Clulow and Jones, 1982; Aire and Ozegbe, 2007) and pass through the excurrent ductus extremely rapidly (1 to 2 d), (Amir et al., 1973; Clulow and Jones, 1982). However, some post-testicular sperm maturation can appear in the epididymis and is manifested in the ability to display progressive motility sufficient to achieve fertilization following natural mating (Nixon et al., 2014). In our study, the secretion of albumin was detected in the epididymis. Perhaps its function can be related to motility development. This suggestion is supported by the results of in vitro study of turkey semen indicating a positive effect of albumin on sperm motility parameters including the percentage of motile spermatozoa, sperm velocity and linearity after short term storage (Bakst and Cecil, 1992).

The results of immunohistochemical localization of albumin within the reproductive tract showed similar sites of albumin secretion in tissues from YSS and WS turkeys, however, with a reduced signal in YSS turkeys. The lesser intensity of staining in YSS male reproductive tissues was unexpected due to results of RT-PCR and western blot analysis. We believe that the main reason for less intensive staining is related to presence of lipid-like vacuoles, present in high amounts in the reproductive tract of YSS turkeys (Hess et al., 1982; Thurston and Korn, 1997), which could have contributed to the formation of unstained vacuoles visible in YSS epididymis (Figure 2D') and ductus deferens (Figure 2F'). Disturbance in lipid metabolism in YSS semen was also confirmed in our recent metabolomic analysis of white and yellow seminal plasma (Słowińska et al., 2018). In summary, disturbance in lipid metabolism may contribute to the numerous lipid vacuoles observed in the reproductive tracts of YSS males which contributed to disturbance in immunohistochemistry. Ductal abnormalities, particularly visible in the epididymis and ductus deferens, presumably impaired the immunohistochemical pattern of albumin localization.

In conclusion, our results demonstrated that cells in the reproductive tract of turkeys synthesized and secreted albumin. The ductus deferens secretion of albumin seems to be mostly responsible for YSS. Oversecretion by the ductus deferens seems to be the main reason for albumin abundance in YSS semen. Disturbance in lipid metabolism may contribute to the numerous lipid-like vacuoles observed in the reproductive tracts of YSS males, which may have caused problems with albumin identification using immunohistochemical techniques.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

REFERENCES

Aire, T. A. 2007. Spermatogenesis and testicular cycles. Pages 1047–1064 in Reproductive Biology and Phylogeny of Birds. B. G. M. Jamieson, ed. Science Publisher, Enfield, NH, USA.

Aire, T. A., and P. C. Ozegbe. 2007. The testicular capsule and peritubular tissue of birds: morphometry, histology, ultrastructure and immunohistochemistry. J. Anat. 210:731–740.

Amir, D., B. Braun-Eilon, and H. Schindler. 1973. Passage and disappearance of labelled spermatozoa in the genital tract of the male Japanese quail in segregation or cohabitation. Ann. Biol. Anim. Bioch. Biophys. 13:321–328.

Arroteia, K. F., M. F. Barbieri, G. H. Souza, H. Tanaka, M. N. Eberlin, S. Hyslop, L. E. Alvares, and L. A. Pereira. 2014. Albumin is synthesized in epididymis and aggregates in a high molecular mass glycoprotein complex involved in sperm-egg fertilization. PLoS One. 9:e103566.

Bakst, M. R., and H. C. Cecil. 1992. Effect of bovine serum albumin on motility and fecundity of turkey spermatozoa before and after storage. J. Reprod. Fertil. 94:287–293.

Bansal, A. K., and G. S. Bilaspuri. 2011. Impacts of oxidative stress and antioxidants on semen functions. Vet. Med. Int. ID 686137, 2011:7.

Bian, Z. H., J. Zhang, X. L. Ding, B. Zhang, Z. J. Wang, C. C. Lu, L. Song, S. L. Wang, and X. R. Wang. 2009. Localization of epididymal protease inhibitor in adult rat and its transcription profile in testis during postnatal development. Asian J. Androl. 11:731–739.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dyebinding. Anal. Biochem. 72:248–254.

Burrows, W. H., and J. P. Quinn. 1937. The collection of spermatozoa from the domestic fowl and turkey. Poult. Sci. 16:19–24.

Clulow, J., and R. C. Jones. 1982. Production, transport, maturation, storage and survival of spermatozoa in the male Japanese quail, *Coturnix coturnix*. J. Reprod. Fertil. 64:259–266.

Deviche, P., L. L. Hurley, and H. B. Fokidis. 2011. Avian testicular structure, function, and regulation. Pages 27–70 in Hormones and Reproduction of Vertebrates. D. O. Norris, and K. H. Lopez, eds. Elsevier Inc., London, UK.

Dietrich, M. A., M. Adamek, B. Bilińska, A. Hejmej, D. Steinhagen, and A. Ciereszko. 2014. Characterization, expression and antibacterial properties of apolipoproteins A from carp (*Cyprinus carpio* L.) seminal plasma. Fish Shellfish Immunol. 41:389–401.

Elzanaty, S., J. Erenpreiss, and C. Becker. 2007. Seminal plasma albumin: origin and relation to the male reproductive parameters. Andrologia 39:60–65.

Esponda, P. 1991. Spermatozoon maturation in vertebrates with internal fertilization. Microsc. Electron. Biol. Celular. 15:1–23.

Go, K. J., and D. P. Wolf. 1985. Albumin-mediated changes in sperm sterol content during capacitation. Biol. Reprod. 32:145–153.

Hejmej, A., M. Gorazd, K. Kosiniak-Kamysz, B. Wiszniewska, J. Sadowska, and B. Bilińska. 2005. Expression of aromatase and estrogen receptors in the reproductive tissues of the stallion and a single cryptorchid visualised by means of immunohistochemistry. Domest. Anim. Endocrinol. 29:534–547.

Hess, R. A., and R. J. Thurston. 1977. Ultrastructure of epithelial cells in the epididymal region of the turkey (Meleagris gallopavo). J. Anat. 124:765–778.

Hess, R. A., and R. J. Thurston. 1984a. Detection and incidence of yellow turkey semen on commercial breeder farm. Poult. Sci. 63:2084–2086.

Hess, R. A., and R. J. Thurston. 1984b. Protein, cholesterol, acid phosphatase and aspartate aminotransaminase in the seminal plasma of turkeys (Meleagris gallopavo) producing normal white or abnormal yellow semen. Biol. Reprod. 31:239–243.

Hess, R. A., G. P. Birrenkott, Jr., and R. J. Thurston. 1984. Seminal plasma androgen-binding protein activity in turkeys with normal white or abnormal yellow semen. J. Reprod. Fertil. 71:403–409.

Hess, R. A., R. J. Thurston, and H. V. Biellier. 1976. Morphology of the epididymal region and ductus deferens of the turkey (Meleagris gallopavo). J. Anat. 122:241–252.

Hess, R. A., R. J. Thurston, and H. V. Biellier. 1982. Morphology of the epididymal region of turkeys producing abnormal yellow semen. Poult. Sci. 61:531–539.

Holsberger, D. R., C. D. Rice, and R. J. Thurston. 2002. Localization of a proteolytic enzyme within the efferent and deferent duct epithelial cells of the turkey (Meleagris gallopavo) using immunohistochemistry. Biol. Reprod. 67:276–281.

King, L. M., D. R. Holsberger, and A. M. Donoghue. 2000. Correlation of CASA velocity and linearity parameters with sperm mobility phenotype in turkeys. J. Androl. 21:65–71.

Kotłowska, M., G. Dietrich, M. Wojtczak, H. Karol, and A. Ciereszko. 2007. Effects of liquid storage on amidase activity, DNA fragmentation and motility of turkey spermatozoa. Theriogenology 67:276–286.

Kotłowska, M., R. Kowalski, J. Glogowski, J. Jankowski, and A. Ciereszko. 2005. Gelatinases and serine proteinase inhibitors of seminal plasma and the reproductive tract of turkey (*Meleagris gallopavo*). Theriogenology 63:1667–1681.

Kotula-Balak, M., R. Zielińska, J. Glogowski, R. K. Kowalski, B. Sarosiek, and B. Bilińska. 2008. Aromatase expression in testes of XY, YY, and XX rainbow trout (*Oncorhynchus mykiss* Walbaum). Comp. Biochem. Physiol. Part. A 149:188–196.

Labas, V., I. Grasseau, K. Cahier, A. Gargaros, G. Harichaux, A. P. Teixeira-Gomes, S. Alves, M. Bourin, N. Gérard, and E. Blesbois. 2015. Qualitative and quantitative peptidomic and proteomic approaches to phenotyping chicken semen. J. Proteomics 112:313–335.

Lemoine, M., I. Grasseau, J. P. Brillard, and E. Blesbois. 2008. A reappraisal of the factors involved in in vitro initiation of the acrosome reaction in chicken spermatozoa. Reproduction 136:391–399.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.

Morris, S. A., B. Howarth, Jr., J. W. Crim, A. Rodriguez de Cordoba, P. Esponda, and J. M. Bedford. 1987. Specificity of sperm-binding Wolffian duct proteins in the rooster and their persistence on spermatozoa in the female host glands. J. Exp. Zool. 242:189–198.

Nixon, B., K. A. Ewen, K. M. Krivanek, J. Clulow, G. Kidd, H. Ecroyd, and R. C. Jones. 2014. Post-testicular sperm maturation and identification of an epididymal protein in the Japanese quail (*Coturnix coturnix japonica*). Reproduction 147:265–277.

Pardyak, L., A. Kaminska, M. Brzoskwinia, A. Hejmej, M. Kotula-Balak, J. Jankowski, A. Ciereszko, and B. Bilinska 2018. Differences in aromatase expression and steroid hormone concentrations in the reproductive tissues of male domestic turkeys (*Meleagris gallopavo*) with white and yellow semen. Br. Poult. Sci. 2:1–13.

Sexton, T. J. 1976. Studies on the dilution of turkey semen. Br. Poult. Sci. 17:179–184.

Shaha, C., C. Y. Cheng, D. M. Phillips, G. P. Talwar, and C. W. Bardin. 1988. Localization of immunoreactive testibumin in the testis and epididymis of adult rats. Int. J. Androl. 11:547–559.

Skinner, M. K, L. Dean, K. Karmally, and I. B. Fritz. 1987. Rete testis fluid (RTF) proteins: purification and characterization of RTF albumin. Biol. Reprod. 37:135–146.

Słowińska, M., E. Liszewska, G. J. Dietrich, and A. Ciereszko. 2012. Characterization of proacrosin/acrosin system after liquid storage and cryopreservation of turkey semen (*Meleagris gallopavo*). Theriogenology 78:1065–1077.

Słowińska, M., E. Liszewska, J. J. Nynca, J. Bukowska, A. Hejmej, B. Bilińska, J. Szubstarski, K. Kozłowski, J. Jankowski, and A. Ciereszko. 2014. Isolation and characterization of ovoinhibitor, a multidomain kazal-like inhibitor from turkey (*Meleagris gallopavo*) seminal plasma. Biol. Reprod. 91:1–15.

Słowińska, M., G. J. Dietrich, E. Liszewska, K. Kozłowski, J. Jankowski, and A. Ciereszko. 2013. Effect of dialysis on the proacrosin/acrosin system and motility of turkey (*Meleagris gallopavo*) spermatozoa during liquid storage. Br. Poult. Sci. 54:661–668.

Słowińska, M., H. Sallem, M. R. Clench, and A. Ciereszko. 2018. Metabolomic analysis of white and yellow seminal plasma in turkeys (*Meleagris gallopavo*). Poult. Sci. 97:1059–1065.

Słowińska, M., J. Bukowska, A. Hejmej, B. Bilińska, K. Kozłowski, J. Jankowski, and A. Ciereszko. 2015b. Hepatocyte growth factor activator is a potential target proteinase for Kazal-type inhibitor

in turkey (*Meleagris gallopavo*) seminal plasma. Theriogenology 84:425–436.

Słowińska, M., J. Jankowski, G. J. Dietrich, H. Karol, E. Liszewska, J. Glogowski, K. Kozłowski, K. Sartowska, and A. Ciereszko. 2011. Effect of organic and inorganic forms of selenium in diets on turkey semen quality. Poult. Sci. 90:181–190.

Słowińska, M., J. Nynca, G. J. Arnold, T. Fröhlich, J. Jankowski, K. Kozłowski, A. Mostek, and A. Ciereszko. 2017. Proteomic identification of turkey (*Meleagris gallopavo*) seminal plasma proteins. Poult. Sci. 96:3422–3435.

Słowińska, M., K. Kozłowski, J. Jankowski, and A. Ciereszko. 2015a. Proteomic analysis of white and yellow seminal plasma in turkeys (*Meleagris gallopavo*). J. Anim. Sci. 93:2785–2795.

Thurston, R. J., and N. Korn. 1997. Semen quality in the domestic turkey: the yellow semen syndrome. Avian Poult. Biol. Rev. 8:109–121.

Thurston, R. J., N. Korn, D. P. Froman, and A. B. Bodine. 1993. Proteolytic enzymes in seminal plasma of domestic turkey (*Meleagris gallopavo*). Biol. Reprod. 48:393–402.

Thurston, R. J., R. A. Hess, and N. Korn. 1992. Seminal plasma protein concentration as a predictor of fertility and hatchability in large white domestic turkeys. J. Appl. Poult. Res. 1:1–4.

Thurston, R. J., R. A. Hess, D. P. Froman, and H. V. Biellier. 1982. Elevated seminal plasma protein: a characteristic of yellow turkey semen. Poult. Sci. 61:1905–1911.

Wishart, G. J., and A. J. Horrocks. 2000. Fertilization in birds. Pages 193–217 in Fertilization in Protozoa and Metazoan Animals: Cellular and Molecular Aspects. J. J. Tarin, and A. Cano, eds. Springer-Verlag, Berlin, Germany.

Zhao, S., and R. D. Fernald. 2005. Comprehensive algorithm for quantitative real-time polymerase chain reaction. J. Comput. Biol. 12:1047–1064.