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1 **Eggshell matrix proteins OC-116, OC-17 and OCX36 in hen's sperm storage tubules**

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9

10 ABSTRACT

11 While uterine epithelium secretes eggshell matrix proteins to regulate eggshell structural
12 organization, uterovaginal junction (UVJ) epithelium supports sperm storage in tubules
13 (SST). Here, we examined the presence of OCX36, OC-116 and OC-17 eggshell matrix
14 proteins in SSTs. Two experimental lines of hens displaying either a long (F+ line) or a short
15 (F- line) potential to store sperm were used, before and 24 hours after insemination. Using
16 immunohistochemistry and western blot, we analyzed the presence of OC-116, OC-17 and
17 OCX36 proteins in the SSTs. Using lectin and calcium staining, we examined the presence in
18 SSTs of Gal/GalNAc (Galactose/N-acetylgalactosamine) and Glc/GlcNAc (Glucose/N-
19 acetylglucosamine) glycans, as well as calcium ions. Our results indicate that in both F+ and
20 F- hens, the eggshell matrix proteins OC-116 and OCX36 were identified in SST cells and
21 lumen, in contact with spermatozoa. The OC-17 protein was found associated with calcium in
22 F+ and F- hens, only in the SST lumen 24h after insemination. Glycans Gal/GalNAc and
23 Glc/GlcNAc were found to be more abundant in the apical cytoplasmic area of the SST cells
24 of F+ hens than in that of F- hens after insemination. This is the first report demonstrating the
25 presence in SSTs of the OC-116, OC-17 and OCX36 eggshell matrix proteins, and their
26 concomitant presence with Gal/GalNAc and Glc/GlcNAc glycans, as well as with calcium.
27 Our results suggest that the OC-116, OC-17 and OCX36 eggshell matrix proteins may be
28 involved in sperm storage.

29 **Key words:** sperm storage tubule, eggshell matrix protein, glycoconjugates, fowl hen

1. Introduction

The avian oviduct is the site of fertilization of the ovulated oocytes as well as that of egg formation. In hens subjected to copulation or insemination, fertilization occurs in the infundibulum within approximatively 15 minutes after ovulation (Wishart and Staines, 1999). The deposition of albumen, shell membranes, shell and shell cuticle takes place in the magnum, the isthmus, and the uterus, respectively (Richardson, 1935; Sauveur and de Reviers, 1988). All the compounds required are produced within the hen oviduct by highly specialized glands (Richardson, 1935; Hodges, 1974). The prolonged sperm storage takes place in two anatomical regions, the uterovaginal junction (UVJ) and the infundibulum. The UVJ is considered as the main site of sperm storage at least in poultry species (Fujii, 1963; Gilbert et al., 1968; Tingari and Lake, 1973). In both sites, the storage is located in highly specific structures called sperm storage tubules (SST) (Bobr et al., 1964; Hodges, 1974).

The SST epithelium is structured in two distinct regions that are connected to each other. The upper portion which opens into the uterovaginal lumen composed of ciliated cells, and the 'blind portion' exclusively made-up of non-ciliated cells (Van Krey et al., 1967) (Burke et al., 1972; Tingari and Lake, 1973; Bakst and Bauchan, 2015; Waqas et al., 2016). Non-ciliated SST cells are the functional portion dedicated to sperm storage in the SST epithelium. These secretory cells were reported to release apocrinal secretions and exosomes (Bakst and Bauchan, 2015; Waqas et al., 2016). They were also reported to contain glycogen, lipids, phospholipid, acid phosphatase (Gilbert et al., 1968; Huang et al., 2016), calcium (Holm et al., 2000) and carbonic anhydrase (Holm et al., 1996). Some of these products, like calcium or carbonic anhydrase, were assumed to impact sperm survival (Fujihara and Koga, 1984; Holm and Ridderstrale, 1998; Holm and Wishart, 1998; Holm et al., 2000). The SST lumen is filled with fluid that also contains several essential factors such as acid phosphatase, lipids (Gilbert et al., 1968) and glycans (Bakst and Bauchan, 2016). Glycans were shown to play a major

55 role in the interaction between sperms and oviductal epithelial cells in various species
 56 including mammals (DeMott et al., 1995; Lefebvre et al., 1997; Green et al., 2001; Wagner et
 57 al., 2002; Kadirvel et al., 2012), birds (Bakst and Bauchan, 2016), and insects (Wong et al.,
 58 2008). It was suggested recently that galactose (Gal) and N-acetylgalactosamine (GalNAc)
 59 moieties from SST apocrinal blebs might mediate communications between resident sperm
 60 and SST cells in the hen's oviduct (Bakst and Bauchan, 2016).

61 Uterine tissue, located in proximity to the UVJ is an epithelium composed of ciliated and
 62 non-ciliated cells which invaginate to form dense array tubular glands (Breen and De Bruyn,
 63 1969). Uterine cells of the surface epithelium and tubular glands display an intense and
 64 dynamic secretory activity, leading to the production of uterine fluid which contains all the
 65 mineral and organic precursors required for shell formation (Gautron et al., 1997). For
 66 example, the uterine fluid is hypersaturated in calcium and bicarbonate ions (Nys et al., 1991),
 67 generated by carbonic anhydrase to produce the CaCO_3 mineral components of eggshells. The
 68 organic phase of the uterine fluid is composed of proteins, glycoproteins and proteoglycans
 69 associated with minerals during the course of shell formation (Gautron et al., 1997; Marie et
 70 al., 2015). Several proteins constitutive of the matrix proteins involved in the processes of
 71 shell mineralization are specifically secreted by uterine cells. Such proteins known as OC-17,
 72 OCX36 and OC-116 are the main actors of shell mineralization (Hincke et al., 1995; Hincke
 73 et al., 1999; Mann and Siedler, 1999; Fernandez et al., 2001; Reyes-Grajeda et al., 2004;
 74 Gautron et al., 2007). These eggshell matrix proteins are involved in calcium aggregation
 75 (Gautron et al., 1997) and calcium precipitation (Gautron et al., 1997; Dominguez-Vera et al.,
 76 2000) within the uterine fluid. OC-17 is a C-type, lectin-like phosphoprotein of 17 kDa
 77 present in glycosylated (23 kDa) and non-glycosylated forms in the shell matrix (Hincke et
 78 al., 1995). It has been related to eggshell strength (Sun et al., 2013). OCX36 is an eggshell
 79 protein belonging to the superfamily of lipopolysaccharide-binding proteins/bactericidal

80 permeability-increasing proteins and Plunc proteins (Gautron et al., 2007). Both proteins play
 81 an antimicrobial role which was clearly established recently (Gautron et al., 2007; Wellman-
 82 Labadie et al., 2008). OC-116 is a protein core of 120-200 kDa eggshell dermatan sulfate
 83 proteoglycan termed ovoglycan which is present in the compact calcified eggshell (Hincke et
 84 al., 1999).

85 In hens, the sperm filling/emptying processes in SST is dependent on egg passage through
 86 the oviduct (Brillard et al., 1987). Moreover, it was suggested that some regulators of eggshell
 87 quality may interfere with *in vivo* sperm storage (Bilgili et al., 1984). In the present study, we
 88 hypothesized that sperm storage in the uterovaginal SST is related to the process of shell
 89 mineralization. We used two lines of hens previously selected for their high or low capacity to
 90 preserve sperm and to produce hatched chicks (Brillard et al., 1998). After determining the
 91 duration of the fertile period in each line, we compared the expression of OCX36, OC-17,
 92 OC-116 secreted proteins and sugar residues in their uterine fluid, SST cells and lumen, and
 93 studied the interaction between SST secretory products and sperm.

95 2. Materials and methods

96 2.1. Birds

97 Sexually mature hens issued from two lines previously selected for their ability to produce
 98 high (F+) or low (F-) numbers of hatched chicks, and to preserve sperm in their SST for
 99 longer (F+) or shorter (F-) periods, were used in this study. These two lines were thus
 100 different in the duration of their fertile period, the F- line expressing a shorter time potential to
 101 lay fertile eggs than the F+ line (Beaumont et al., 1992). Breeding procedures and handling
 102 protocols were carried out according to the European Community Council Directives
 103 regarding the practice for care and use of animals and of the recommendations of the French
 104 Ministry of Agriculture on Animal Experimentation under the supervision of an authorized

105 scientist (Authorization # 37035). Farm facilities (Institut National de la Recherche
106 Agronomique, UE-PEAT, 1295) are recognized as officially authorized to rear and euthanize
107 birds (B27-175-1 dating back to August 28th, 2012). At 43 wks of age, hens were placed in
108 individual cages equipped with automatic devices to record the time of oviposition. They
109 were kept under a 16L: 8D photoperiod, and fed a layer mash *ad libitum*.

110 2.2. Fertility Rates and Duration of Fertile Period

111 The duration of the fertile period within each line was expressed using two definitions:
112 effective duration (De), the number of days after artificial insemination (AI) during which a
113 hen lays 100 % fertile eggs; and maximum duration (Dm), the number of days after AI until
114 the hen lays its last fertile egg. At 45 wks of age, F+ (n=28) and F- (n=27) hens were
115 inseminated on two consecutive days with 200×10^6 sperm from mixed ejaculates collected
116 from 6 broiler breeder males on both days. The eggs were identified individually and recorded
117 daily from Day 2 to Day 22 following the second AI. They were stored for a maximum of 7
118 days prior to incubation. The percentage of fertile eggs was determined by candling on Day 7
119 of incubation. Eggs primarily classified as infertile were broken up for macroscopic
120 examination of the germinal disc to determine the presence of dead blastodiscs, if any.
121 Comparisons for De, Dm, and fertility, laying and embryo mortality rates between the two
122 lines were computed using a Mann-Whitney test ($P < 0.05$).

123 2.3. Collection of uterine fluid and sperm incubation

124 The collection of uterine fluid (UF) was planned individually 10 hours after a naturally
125 achieved oviposition, from F+ (n=4) and F- (n=4) hens that display high and similar laying
126 rates (0.8-1 egg/day). The presence of a forming egg *in utero* was confirmed and its expulsion
127 from the uterus was carried out by intravenous injection of 50µg of prostaglandin F2α. During

egg expulsion, the UF was collected by abdominal massage into a plastic tube placed at the opening of the everted vagina (Gautron et al., 1997).

Spermatozoa from 6 broiler breeder males were rinsed 3 times in BPSE diluent (10 min, 600g). They were incubated at 41°C during 1 hour in UF collected from F- or F+ hens at a final concentration of 1×10^6 sperm/mL. Spermatozoa were rinsed in PBS and fixed in 4% saline buffered (PBS) formalin before immunohistochemistry procedure.

2.4. *Tissue Collection*

Tissue samples of the uterus, vagina and uterovaginal junction (UVJ) were collected for histochemistry between 8-10 hours after oviposition. Hens that display high and similar laying rates (0.8-1 egg/day) were euthanized by cervical dislocation, either before AI (n=3 for each line) or 24 hours after AI (n=3 for each line). The whole oviduct from uterus to vagina was excised as one segment. Connective tissue was removed to expose the UVJ. Samples of uterus, vagina and UVJ mucosa containing SST were collected from each hen and processed.

2.5. *Western blotting*

The protein concentration was determined in each UF sample individually, by the Pierce® BiCinchoninic Acid protein assay kit (Life Technologies SAS, Saint Aubin, France) using bovine serum albumin as the protein standard and according to the manufacturer's instructions. Ten µg of UF proteins were added to 5x Laemmli buffer (5v:1v), boiled at 95°C for 5 min, then loaded and separated on a 10% SDS-PAGE, before being transferred to nitrocellulose membranes. The membranes were washed with TBS (10mM Tris, 150mM NaCl, pH 7.4) containing 0.1% (v/v) Tween-20 (TBS-T), incubated for 1 hour in the blocking solution (5% (w/v) non-fat dry milk in TBS-T), and overnight in the blocking solution containing anti-OC-17, anti-OC-116, or anti-OCX36 (1:1000 each). All antibodies were polyclonal produced in the rabbit and kindly provided by J. Gautron (Unité de Recherches

Avicoles, INRA, Nouzilly, France). The membranes were then sequentially washed with TBS-T, incubated for 1 hour in the blocking solution, then for 1 hour with peroxidase-conjugated secondary antibody, i.e. goat anti-rabbit IgG (A6154, Sigma-Aldrich, Saint Quentin Fallavier, France) diluted 1:5000 in the blocking solution, and finally washed with TBS-T. The peroxydase activity was detected with the ECL select™ Western Blotting Detection Reagent (GE Healthcare, Velizy-Villacoublay, France), the signal was captured by mean of an ImageMaster VDS-CL bio imaging system (Amersham Biosciences/GE Healthcare) and quantified using Image Quant software. Signal was then normalized on the total loaded protein quantity (Ponceau red).

2.6. *Histochemistry Preparation for Immuno and Lectin Detection*

Following 24 hours fixation in 4% saline buffered (PBS) formalin, tissue samples (uterus, vagina and UVJ) were transferred to two successive bathes of 70% ethanol (2x30 min), and then moved to an automated system tissue processor (Leica TP1020 Semi-enclosed Benchtop Tissue Processor). The samples were then embedded in paraffin (Leica EG1150 Modular Tissue Embedding Center, Leica Microsystems Richmond, Inc., Richmond, IL). Four to six 7- μ m thick sections were collected in sequence onto slides treated with albumin, air-dried overnight, stored at room temperature (RT) for 12 hours and then incubated overnight at 60°C. The staining procedures consisted in deparaffinization for 5 min in toluene, followed by progressive rehydration (100%, 95%, 80% and 70% ethanol bathes, 2 min each) and removal of ethanol excess by water bathes.

2.7. *Immunohistochemistry*

After dehydration, the slides were treated with 1% citrate based unmasking solution H3300 (Vector Laboratories, Burlingame, CA) diluted as recommended by the manufacturer. The slides were rinsed once in TBS for 5 min and then placed in a bath with normal horse serum

176 blocking solution (Vector) for 20 min. The slides were incubated overnight at 4°C with the
 177 primary antibodies, anti-OC-17 (1:500), anti-OC-116 (1:500) or anti-OCX36 (1:500) diluted
 178 in TBS with 5% non-fat dry milk. The slides were then washed 3 times in TBS (5 min), and
 179 incubated for 30 min with the secondary antibody ImmPRESS™ HRP Anti-Rabbit/Mouse
 180 IgG (Vector) and rinsed in TBS (5 min), incubated with peroxidase ImmPACT NovaRED
 181 (Vector) and finally rinsed in distilled water (5 min). Sections were counterstained with
 182 Papanicolaou stain (Sigma). Coverslips were applied with aqueous mounting medium after
 183 dehydration through graded alcohol bath (70%, 80% 95%, 100%; 20 sec each) and incubation
 184 of the sections in toluene (1 min). The sections were examined using an Axioplan Carl Zeiss
 185 microscope. Images were acquired using a digital monochrome camera (Spot-Flex,
 186 Diagnostic Instruments) coupled with the SPOT 5.2 imaging software. The same procedure
 187 was used for negative control of the slides with an exception for the first antibody. Negative
 188 controls for immunohistochemistry demonstrated the absence of unspecific signal making the
 189 procedure reliable.

190 2.8. *Von Kossa staining*

191 Calcium deposits in the UVJ tissue were detected using the Von Kossa staining method.
 192 Briefly, deparaffinized and rehydrated sections were incubated with 2% silver nitrate solution.
 193 The slides were exposed to UV for 20 min until a light brown signal appeared, then rinsed in
 194 distilled water before removal of unreacted silver with a 5% sodium thiosulfate solution (5
 195 min). The sections were rinsed several times in water, counterstained with Papanicolaou
 196 staining, gradually dehydrated (in 70%, 80%, 90% and 100% ethanol, for 2 min each) and
 197 cleared in toluene (5 min). The slides were permanently covered with Depex® aqueous
 198 mounting medium. Calcium deposits were observed as a light to dark brown signal.

199 2.9. *Lectin Histochemistry*

200 Lectins (Vector Laboratories, Burlingame, CA) used in this study are reported in Table 1.
 201 For each set of slides processed, two negative controls were prepared: one slide was incubated
 202 without lectin to detect auto-fluorescing cells, and another slide was incubated in the presence
 203 of lectin and of its inhibiting sugar for at least 40 min. All slides were incubated for 10 min in
 204 TBS prior to tissue labeling with a single lectin at its optimized concentration for 60 min
 205 (Table 1). The slides were transferred 3 times for 10 min in TBS, and then washed for 3 min
 206 in deionized water. This procedure was repeated for dual lectin labeling. Coverslips were
 207 applied with Fluoromount-G® (Southern Biotech, USA) mounting medium. The slides were
 208 protected from light during the process of fluorescence conjugated-lectin labelling. Then, the
 209 slides were dried in the dark at room temperature for 24 hours and stored at 4°C until analysis.
 210 Imaging was completed within one week after labeling. The sections were examined using a
 211 Zeiss LSM 700 confocal microscope equipped with the Zen 2012 64-bit image acquisition
 212 and processing software. The negative control procedure for lectin histochemistry
 213 demonstrated the absence of unspecific signal, making the procedure reliable for sugar
 214 detection.

215

216 3. Results

217 3.1. *Fertility characteristics of F+ and F- hens*

218 As shown in Figure 1 and Table 2, a highly significant difference was recorded in most
 219 fertility parameters between the two selected lines, with up to a 27.8% increase in egg fertility
 220 rate between F+ hens and F- hens ($P < 0.001$). Moreover, the fertile period of F+ hens
 221 exceeded that of F- hens by 1.9 and 6.0 days for De and Dm, respectively ($P < 0.05$ and $P <$
 222 0.001). Accordingly, the mean percentage of fertile eggs was constantly higher in F+ than in
 223 F- eggs over the three weeks after insemination. The mean differences in fertile eggs were
 224 19.7, 53.2 and 13.9 % during weeks 1, 2 and 3, respectively ($P < 0.01$, $P < 0.001$ and $P <$

0.001). Moreover, the laying rates were significantly higher in F+ hens than in F- hens ($P < 0.01$) over the experimental period. No significant difference between the two lines was recorded in the percentage of embryo survival, irrespective of the week following insemination.

3.2. *OC-116, OC-17 and OCX36 in uterine fluid (UF)*

OC-116, OC-17 and OCX36 were detected by Western blot in UF at 116kDa, 36kDa and 17kDa, respectively (Figure 2A). As shown in Figure 2B, the signals were stronger for F+ hens than for F- hens, even though the difference is not significant. Nevertheless, the immuno-detection signals of all the three proteins tended to increase after AI in F- hens, whereas they tended to decrease in F+ hens.

3.3. *OC-116, OC-17 and OCX36 immunohistochemistry in vagina and uterus*

We observed that the epithelium of the vagina and uterus showed different affinities for OC-116, OC-17 and OCX36 antibodies. None of the 3 proteins were detected in the vagina epithelium (Figures 3A-C). OC-116 was localized in the uterine surface epithelium (Figure 3E) while OC-17 was localized exclusively in the uterine glandular epithelium (Figure 3F). OCX36 was detected neither in the uterine epithelium, nor in the uterine glandular epithelium (Figure 3G). Negative controls for immunohistochemistry demonstrated the absence of unspecific signal in vagina (Figure 3D) and uterus (Figure 3H).

3.4. *OC-116, OC-17 and OCX36 immunohistochemistry in UVJ*

3.4.1. *OC-116 protein*

OC-116 was localized in UVJ surface epithelial cells and in SST cells cytoplasm (Figures 4A-D). Moreover, in both F+ and F- hens, the signal appeared luminal even before AI (Figures 4A, 4C, 4C inset) or after AI (Figures 4B, 4B inset i, 4D, 4D insets iii and v). Interestingly after AI, the luminal staining was in contact with spermatozoa in F- hens (Figure

4B inset ii) and surrounded spermatozoa in F+ hens (Figure 4D insets v and vi). Moreover, we observed a granular secretory product with an intense staining for OC-116 projecting outside SST (Figure 4D insets iii and iv). OC-116 displayed a crown-shaped signal around spherical structures localized inside SST (Figure 4D inset vii).

3.4.2. OC-17 protein

OC-17 has never been reported in UVJ tissue, neither in the epithelium nor in SST cells, whatever the genetic line, before or after AI (Figure 5). Twenty-four hours after AI, some signal for OC-17 was localized in the lumen of SST in both F- (Figure 5B inset), and F+ (Figure 5G inset i) hens. OC-17 reactivity co-localized with Von Kossa calcium staining (Figure 5G inset ii). This co-staining was observed in SST filled with spermatozoa (Figure 5D insets i and ii). Negative controls for immunohistochemistry demonstrated the absence of unspecific signal in UVJ from F- (Figure 5C) and F+ (Figure 5H) hens after AI, as well as in sperm incubated with UF collected from F- (Figure 5D) and F+ (Figure 5I) hens. No reactivity was detected in sperm incubated with UF collected from F- (Figure 5E) and F+ (Figure 5J) hens.

3.4.3. OCX36 protein

In UVJ, no signal for OCX36 was detected in surface epithelial cells, but was specifically observed in SST cells from both F+ and F- hens, either without AI or 24h after AI (Figures 6A-D). Without AI, the signal was heterogeneous, and located in the cytoplasm of SST cells in both F- (Figure 6A) and F+ (Figure 6C) hens. Moreover, a granular signal was also observed projecting in the lumen of SST from F- (Figure 6A insets i and ii) and F+ hens (Figure 6C inset). After AI, the localization of OCX36 was mainly luminal in F- hens, with only a small amount observed in the cytoplasm of few cells (Figure 6B). Some spermatozoa appeared enclosed in a substance positive for OCX36 staining (Figure 6B inset). In contrast,

in F+ hens after AI, the OCX36 signal was very strong within the cytoplasm all along the SST (Figure 6D).

3.5. *Lectin histochemistry*

3.5.1. *Lactosamine (Gal β 1- $\rightarrow$ 4GlcNAc), Terminal N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc) residues in the vagina and uterus*

As shown in Figure 7, we observed that in vaginal and uterine tissues, ECL and WGA bound with high affinity the Gal β 1- $\rightarrow$ 4GlcNAc and the GlcNAc residues, respectively. Moreover, SBA bound with low affinity the GalNAc residues from both tissues. In vagina, ECL staining was strong in the cytoplasm of some epithelial cells, as well as in loose connective tissue (Figure 7A). Moreover, SBA lectin bound to lymphocytes of loose connective tissue (Figure 7B), and WGA bound with strong affinity to the cytoplasmic part of some epithelial cells (Figure 7C). Negative controls for lectin histochemistry demonstrated the absence of unspecific signal in vagina (Figure 7D). In uterus, ECL staining was demonstrated in the cytoplasmic and the apical part of surface epithelial cells (Figure 7E), and of tubular gland cells (Figure 7E inset, arrowhead). SBA lectin bound to lymphocytes of loose connective tissue, as previously observed in the vagina (Figure 7F). WGA strongly bound to the apical part of uterine surface epithelial cells as well as that of tubular gland cells (Figure 7G inset, arrowhead), and with less intensity to their cytoplasmic compartment (Figure 7G). Negative controls for lectin histochemistry demonstrated the absence of unspecific signal in uterus (Figure 7H).

3.5.2. *N-acetylglucosamine (GlcNAc) residues in UVJ*

In UVJ, WGA staining differed between F+ and F- hens both in non-inseminated and inseminated females (Figure 8). In non-inseminated F- hens WGA staining was apparent in the cytoplasm of some surface epithelial cells and SST cells, although irregular, less

homogenous and stronger in the former (Figure 8A). In non-inseminated F+ hens WGA staining was apparent in the apical part of surface epithelial cells and SST cells, although more homogenous and stronger in the latter (Figure 8C). Of note is the differential WGA staining of the apical region of UVJ cells from F+ and F- hens. After AI, WGA displayed specific staining patterns. In F- UVJ tissue, it appeared strongly bound to the apical and supranuclear part of surface epithelial cells and SST cells (Figure 8B), whereas in F+ UVJ tissue, it strongly highlighted the cytoplasmic part of some surface epithelial cells, as well as the apical part of SST cells (Figure 8 inset i). This staining was associated with a strong staining of the SST lumen too (Figure 8D inset ii). Of note was the absence of WGA staining within the F+ SST cells concomitant with an intense staining of SST cell apex (Figure 8D).

3.5.3. *Lactosamine (Gal β 1- $\rightarrow$ 4GlcNAc) residues in the UVJ*

In UVJ, ECL binding differed between F+ and F- hens, both in non-inseminated and inseminated females (Figure 9). In non-inseminated F-, it appeared bound irregularly and strongly to cytoplasmic and apical parts of some surface epithelial cells and SST cells (Figure 9 A inset i and ii). In non-inseminated F+ hens, it appeared bound regularly to the apical part of epithelial and SST cells (Figure 9C). Moreover, ECL staining was visualized in loose connective tissue from F- UVJ tissue, but not in that from F+. After AI, ECL displayed specific staining patterns. In F- UVJ tissue, it homogeneously bound to the top of cilia from the surface epithelium (Figure 9B, arrowhead), as well as to the apical and the cytoplasmic parts of SST cells (Figure 9B inset iii). Moreover, some SST cells showed an intense ECL staining. We also observed that the SST lumen displayed ECL staining (Figure 9B inset iv). Furthermore, the F- UVJ loose and dense connective tissue was strongly stained by ECL lectin. In F+ hens after AI, ECL staining was observed irregular and strong in cytoplasmic and apical parts of some surface epithelial cells (Figure 9D inset vi) and some SST cells

(Figures 9D encircled, 9D inset v). Furthermore, it is of note that in both F+ and F- UVJ collected after AI, some SST cells were strongly stained by ECL lectin (Figures 9 B-D).

3.5.4. Terminal N-acetylgalactosamine (GalNAc) residues in the UVJ

The staining density of SBA lectins appeared similar in the in UVJ tissues of F+ and F- hens (Figure 10). In non-inseminated hens, SBA lectin was bound the cytoplasmic and apical parts of SST cells (Figures 10 A-D). After insemination, the SBA staining was localized to the top of cilia from epithelium as a thick layer (Figures 10B, 10B inset i and Figure 10D inset, arrowheads). Moreover, the SBA staining displayed a stronger signal at the apical part of SST cells (Figures 10B-D) and lumen (Figure 10B inset ii and Figure 10E) than in surface epithelial cells. Interestingly, the intense luminal staining in F+ hens observed in Figure 10D appears to surround the underlying spermatozoa shown in Figure 10E. Moreover, some SST cells are intensively stained with SBA lectin.

4. Discussion

The present work examined the expression of OCX36, OC-17 and OC-116, three glycoproteins known to participate to the eggshell calcification, within SSTs in which sperm is stored for quite a long period of time. Actually, we hypothesized that sperm storage within SSTs and the eggshell mineralization process by the uterus are related.

Using two lines of hens (F+ and F-), we showed that the genetic divergence on the duration of fertility remains as originally described (Brillard et al., 1998). The F- hens are characterized by a reduced fertility, a reduced laying rates, as well as shorter efficient and maximal durations of fertility when compared to the F+ line. Fertilization rates after the first, the second and the third week post-insemination were also significantly reduced in F- hens, in

344 comparison to F+ hens, as previously described (Brillard et al., 1998). Therefore these two
345 lines are a suitable model to study the regulation of sperm storage duration in hens, and more
346 generally in avian species.

347 We showed for the first time in the present study that SST epithelial cells, but not UVJ
348 surface epithelial cells, express simultaneously eggshell matrix proteins OC-116 and OCX36
349 concomitantly with the presence of sugar residues Gal/GalNAc and Glc/GlcNAc. In addition,
350 this work is the first that show the presence of OC-17 in SST lumen. Nevertheless, we did not
351 observed any OC-17 signal within SST cells during our immunolocalization experiment.
352 Thus, OC-17 may either be secreted as soon as synthesized by SST cells, or less probably, it
353 may originate from uterine tubular glands and then accumulates in SSTs. Of note that OC-17
354 originating from the uterine fluid was never found attached to sperm after *in vitro* incubation.
355 This indicates that OC-17 in SST lumen was not absorbed onto sperm membrane. In the
356 present study, the presence of OC-17, OC-116 and OCX36 was associated with calcium
357 deposits. Considering the role of eggshell proteins on calcium aggregation (Gautron et al.,
358 1997) and eggshell quality (Sun et al., 2013), the present study confirms previous
359 observations showing that eggshell quality regulators interfere with sperm storage (Bilgili et
360 al., 1984). This leads us to hypothesize that eggshell matrix proteins may display important
361 feature for sperm storage.

362 OC-116 is a protein core of a proteoglycan named ovoglycan, composed of dermatan
363 sulfate glycosaminoglycan chains that contain disaccharide units of both N-acetylglucosamine
364 and iduronic acid. It was first identified in eggshell (Carrino et al., 1996; Carrino et al., 1997;
365 Hincke et al., 1999; Fernandez et al., 2001). The precise role of this macromolecule is not
366 clearly known, considering the complexity of the proteoglycan part containing dermatan
367 sulfate (length, localization of L-iduronic acid, sulfation etc.), associated to the core protein
368 specificity. All in all, dermatan sulfate macromolecules are known to have binding

369 capabilities for various extracellular matrix proteins, growth factors (TGFb), protease
 370 inhibitors, cytokines, chemokines, and pathogen virulence factors (Trowbridge and Gallo,
 371 2002). In the context of mineralization, it has been proposed that the negative charges of
 372 acidic ovoglycan confer the capacity to attract divalent ions like calcium (Hincke et al., 1999).
 373 This is supported by recent works demonstrating that glycosaminoglycans can entrap calcium
 374 as nanoparticles (20-60 nm) during crystallization process *in vitro* (Chen et al., 2010; Chen et
 375 al., 2012).

376 In mammals, OC-116 ortholog MEPE encodes the matrix extracellular
 377 phosphoglycoprotein (MEPE), which has been shown to be involved in bone mineralization.
 378 OC-116 and its mammalian orthologue MEPE are part of the Small Integrin-Binding Ligand,
 379 N-linked Glycoprotein (SIBLINGS) family containing non collagenous proteins that are
 380 mainly expressed in mineralized tissues, bone, and dentin (Fisher et al., 2004; Bellahcene et
 381 al., 2008; Bardet et al., 2010). One of the most significant differences between OC-116 and
 382 other SIBLINGS (including mammalian MEPE) is its original identification as a dermatan
 383 sulfate proteoglycan. As such, OC-116 was thought to influence calcitic mineralization, in
 384 part via interaction with sulfate anion (Rose and Hincke, 2009). Nevertheless, OC-116 is also
 385 a major phosphoprotein of the eggshell matrix (Mann et al., 2007), and was recently identified
 386 in young chick cortical bone, laying hen medullary bone and growth plate hypertrophic
 387 chondrocytes suggesting a possible role in calcium phosphate mineralization. Different post-
 388 translational modifications in the OC-116 protein are likely important in determining the
 389 nature and influence of OC-116 interaction with calcium carbonate in eggshell or calcium
 390 phosphate in bone. Although there is no evidence yet for the presence of calcium carbonate or
 391 calcium phosphate in SST lumen, the presence of OC-116 suggests at least the modulation of
 392 SST luminal calcium deposits. Distribution of the two forms of OC-116 (proteoglycan and
 393 phosphoprotein) within SSTs, i.e. phosphorylated, N-glycosylated or glycanated, need to be

394 further investigated. Our study confirms the presence of several intracellular and apical sugar
395 moieties, suggesting that OC-116 could be at least glycosylated in SSTs.

396 Considering its role in calcium modulation, we suggest that OC-116 acts as a calcium
397 chelator regulating OC-116-bathing-sperm motility as well as sperm viability in SSTs. This
398 could be related to previous data demonstrating that the addition of calcium in sperm diluents
399 improves sperm viability (Fujihara and Koga, 1984; Wishart and Ashizawa, 1987; Wishart
400 and Wilson, 1999). Moreover, Froman (2003) proposed a model of sperm behavior to enter
401 and survive in SSTs, based on the role of calcium on sperm motility (Froman, 2003).

402 Ovocleidin-17 (OC-17) is an abundant component of the soluble fraction of the uterine
403 fluid and it is also glycosylated (Mann, 1999). It is one of the major candidates to regulate
404 mineral deposition. Based on its amino acid sequence, OC-17 contains two phosphorylated
405 sites, and a C-type lectin-like domain (Mann, 1999) like other avian homologous eggshell
406 matrices (Mann and Siedler, 2004; Lakshminarayanan et al., 2005; Mann and Siedler, 2006).
407 Of note, C-type lectin-like domain containing proteins are associated with the mineral phase
408 of calcium carbonate.

409 This is the first study reporting the co-localization of OC-17 and calcium positive
410 substance, in relation to sperm storage in SSTs. This co-localization was observed only after
411 insemination, and only in sperm-filled SSTs, suggesting that sperm may regulate the release
412 of calcium from the SST epithelium. Alternatively, this co-localization of OC-17 and calcium
413 suggests a modulation in the SST luminal environment, since OC-17 is known to participate
414 in the crystallization of amorphous calcium carbonate (Reyes-Grajeda et al., 2004). The
415 presence of the OC-17 protein and calcium ions inside SST may illustrate the dynamic
416 activity of cells that may lead to the modulation of the environment to permit sperm retention
417 or release. This is consistent with the fact that eggshell matrix proteins are secreted in variable

quantities by the uterine surface epithelium or tubular glands during shell mineralization (Marie et al., 2015).

One potential role of OC-17 and OCX36 proteins could be to maintain a bacteria free environment in the SST lumen. Indeed, OCX36 belongs to the superfamily of lipopolysaccharide-binding proteins/bactericidal permeability-increasing proteins and Plunc proteins which displays antimicrobial activity (Gautron et al., 2007). The OC-17 protein is a C-type lectin like phosphoprotein that also exhibits antimicrobial properties (Wellman-Labadie et al., 2008).

Interestingly, we recorded differences between the two lines of hens regarding staining intensity and localization of both the Gal/GalNAc and Glc/GlcNAc moieties, 24 hours after insemination. Our observations suggest a higher secretory activity in F+ SST cells after insemination than in F- SST cells. Thus, our results support the importance of glycoconjugates, probably originating from eggshell matrix proteins, on the duration of sperm storage, as proposed recently by Bakst et al. (2016) (Bakst and Bauchan, 2016). Associated with the fact that the localization of Gal/GalNAc and Glc/GlcNAc moieties was different in F+ and F- hens before AI, our results suggest a discrepant sensitivity to insemination of SST cells in the two lines.

Glycan-mediated interactions are known to be involved in the attachment of sperm to the oviduct epithelium in mammalian sperm reservoir (Suarez, 2001). N-acetylglucosamine moieties, as well as galactose moieties, are involved in sperm adherence to epithelial cells (Suarez, 2016). In hen's oviduct, galactose and N-acetylgalactosamine moieties have been recently proposed to mediate sperm attachment to SST epithelial cells in turkey (Bakst and Bauchan, 2016). Galactose and N-acetylgalactosamine moieties can be part of galactosaminoglycans that are anionic polysaccharides covalently attached to core proteins to form proteoglycans. These polysaccharides have been associated to eggshell mineralization in

hen's oviduct (Nakano et al., 2002). Moreover, dermatan sulfate proteoglycan OC-116 is a galactosaminoglycan composed of N-acetylgalactosamine and N-acetylglucosamine residues that combine to form the major motif that is present on the molecule, LacDiNAc (GalNAc β 1-4GlcNAc). Interestingly, OC-116 contains several fucosylated forms of LacDiNAc motif (Nimtz et al., 2004). However, fucose residues are known to be key components of sperm binding to mammalian oviduct (Suarez, 2008). Furthermore, it has been reported that OC-17 contains N-acetylgalactosamine, but not N-acetylglucosamine moieties (Hincke et al., 1995). Therefore, a better understanding of the role of the eggshell matrix proteins (OC-116 and OC-17) and their N-acetylgalactosamine and N-acetylglucosamine moieties is needed in the context of sperm adherence on SST epithelial cells.

In conclusion, this is the first study reporting the presence of OC-116, OCX36 and OC-17 eggshell matrix proteins in SSTs, their concomitant expression with Gal/GalNAc and Glc/GlcNAc, as well as with calcium and sperm. Our results suggest that the storage of sperm in the SSTs and the eggshell mineralization process, assumed by OC-116, OCX36 and OC-17, may be related. In our view, the present study also opens up new research prospects. Thus, the study of the presence of eggshell matrix proteins, glycoconjugates and calcium in infundibulum, specifically in SSTs, should receive interest to better understand their role in avian species like in F⁺ and F⁻ hen genetic lines. Another matter of interest refers to the main biochemical properties of the eggshell matrix proteins which appear well conserved in most avian species and other sauropsids (snakes, crocodiles, turtles) (Panheleux et al., 1999; Bardet et al., 2010). Considering that these species are also known to maintain sperm survival in their genital tract (Holt, 2011), the study of eggshell matrix proteins expression should receive additional attention to better understand their role(s) at maintaining a local environment favoring the long term storage of sperm in SSTs.

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645 Figure legends

646 **Figure 1.** Egg fertility in hens F+ (n=28) (black bars) and F- (n=27) (white bars) during the
 647 first, second and third week after artificial insemination of two 200×10^6 spermatozoa per hen
 648 performed on two consecutive days at 45 weeks of age. Data are expressed as mean $\pm$ SEM.
 649 The differences between the mean values of the two lines were evaluated using a Mann-
 650 Whitney test. Values differed. * $p < 0.01$; ** $p < 0.001$.

651 **Figure 2.** OC116, OCX36 and OC17 amounts in UF from F+ and F- hens. (A) Representative
 652 profiles of OC116, OCX36 and OC17 amounts in UF from F+ and F- hens. (B) Quantitative
 653 analysis of OC-116, OCX36 and OC17 amounts in UF from F+ (n=4) and F- hens (n=4)
 654 without AI or 24h after AI.

655 **Figure 3.** Localization of OC116, OC17 and OCX36 proteins using immunohistochemistry:
 656 vaginal (A, B, C, D) and uterine (E, F, G, H) sections. No reactivity for OC-116, OC-17 and
 657 OCX36 was detected in vagina sections (A-C). OC-116 staining was limited to uterine surface
 658 epithelium (D) while OC-17 staining was limited to uterine tubular glands (E), and OCX36
 659 displayed no reactivity in uterus (F). Negative controls for immunohistochemistry
 660 demonstrated the absence of unspecific signal in vagina (D) and uterine (H) tissue. Figure is
 661 representative of several observations. l, lumen; mf, mucosa fold. Scale bar: A-B-C-D-E-F-G-
 662 H, 20 μ m.

663 **Figure 4.** Immunohistochemistry of OC-116 protein in the UVJ tissue of F- (A,B) and F+
 664 (C,D) hens having not subjected (A,C) or subjected to AI (B,D). (A) F- epithelial cells from
 665 SST as well as UVJ show positive reactivity for OC-116. The lumen of SST is also staining
 666 (arrowhead). (B) F- epithelial cells from SST demonstrate a positive signal for OC-116. Insets
 667 show the OC-116 signal in the lumen of SST after AI (i, arrowhead). The luminal staining (ii,
 668 arrowhead) is in contact with spermatozoa (ii, arrow). (C) F+ SST cells as well as UVJ show

669 positive reactivity for OC-116. Staining is also observed in the lumen of SST (arrowhead).
 670 (D) While F+ SST epithelial cells are positively stained for OC-116, the SST lumen
 671 containing sperm is also stained (arrowhead). A staining of secretion (encircled) projecting
 672 outside SST is observed (iii). The aspect of this secretion is granular (iv). OC-116 signal is
 673 visualized surrounding resident sperm (arrowhead, v and vi). Presence of OC-116 in the
 674 crown region of a substance filling a large SST (arrowhead, vii). Figure is representative of
 675 several observations. l, lumen; SST, sperm storage tubule. Scale bar: A-B-C-D, 20 μ m; insets
 676 in: B-C-D.iv-D.v-D.vi, 10 μ m, insets in: D.iii-D.vii, 20 μ m.

677 **Figure 5.** Immunohistochemistry of OC-17 protein and calcium staining by Von Kossa's
 678 method in the UVJ tissue from F- (A,B) and F+ (F,G) hens without AI (A,F) and 24 hours
 679 after AI (B,G), and in sperm incubated with UF collected from F- (SPZ + UF-) (D, E) and F+
 680 (SPZ + UF+) (I, J) hens. (A, F) No reactivity was observed in UVJ and SST epithelial cells
 681 from F+ and F- hens not subjected to AI. (B, G) SST containing sperm demonstrate no
 682 reactivity for OC-17 after AI. Nevertheless, some large SST show OC-17 luminal positive
 683 staining (arrowhead, inset in B and inset i in G) which is concomitant with the presence of
 684 resident sperm and a positive signal for Von Kossa's staining method (inset ii in G). (C, H)
 685 Negative controls for immunohistochemistry in UVJ from F- and F+ hens after insemination.
 686 (D, I) Negative controls for immunohistochemistry in sperm incubated with UF from F- and
 687 F+ hens. (E, J) No reactivity was detected in sperm incubated with UF collected from F- and
 688 F+ hens. Figure is representative of several observations. l, lumen; SST, sperm storage tubule.
 689 Scale bar: A-B-C-D, 20 μ m; insets in: B-D, 20 μ m.

690 **Figure 6.** Immunohistochemistry of OCX36 protein in the UVJ tissue from F- (A,B) and F+
 691 (C,D) hens without AI (A,C) and 24 hours after AI (B,D). (A) F- epithelial cells from SST,
 692 but not UVJ, were positive for OCX36 immunodetection in hen having not subjected to AI.
 693 Inset shows a granular luminal secretion from SST, positive for OCX36 (i and ii). (B) F- SST

694 epithelial cells display positive signal for OCX36 after AI. SST lumen was also positive for
 695 OCX36 (arrowhead), and resident sperm (arrow) is surrounded by SST secretion positive for
 696 OCX36 (arrowhead). (C) F+ SST epithelial cells as well as lumen (inset) display positive
 697 reactivity for OCX36. (D) In SSTs filled of spermatozoa, SST epithelial cells were
 698 homogeneously positive for OCX36 after AI. Figure is representative of several observations.
 699 l, UVJ lumen; SST, sperm storage tubule. Scale bar: A-B-C-D, 20 μ m; insets in: A-D, 10 μ m.

700 **Figure 7.** Staining of vagina (A,B,C,D) and uterus (E,F,G,H) tissue incubated with ECL
 701 (A,D), SBA (B,E) and WGA (C,F) lectins. (A) Mucosa from vagina demonstrates ECL
 702 positive reactivity. Inset shows the granular aspect of the ECL staining in some epithelial cells
 703 of vaginal mucosa. (B) Mucosa from vagina displays no reactivity for SBA except in
 704 lymphocytes of the loose connective tissue. (C) Surface epithelium from vagina is positive for
 705 WGA immunodetection. Inset shows the apical and the supranuclear WGA staining in cells.
 706 (D) Negative control for lectin histochemistry in vagina. (E) Uterine mucosa is positive for
 707 ECL immunodetection. Inset focalizes on the apical signal observed in surface epithelial cells
 708 and tubular gland cells (arrowhead). (F) No reactivity for SBA is observed in uterine mucosa,
 709 except in lymphocytes of the loose connective tissue. (G) Apical WGA staining of uterine
 710 surface epithelial and tubular gland cells (arrowhead) are observed. (H) Negative control for
 711 lectin histochemistry in uterus. Figure is representative of several observations. l, lumen; mf,
 712 mucosal fold. Scale bar: A-B-C-D-E-F-G-H, 50 μ m; insets in: A-C-E-G, 10 μ m.

713 **Figure 8.** Staining of GlcNAc containing glycans by WGA lectin in the UVJ tissue of F-
 714 (A,B) and F+ (C,D) hens having not been subjected (A,C) or subjected to AI (24h: B,D). (A)
 715 Intracellular staining is detected in the F- UVJ surface epithelial cells as well as in the
 716 transition zone with SST cells. (B) After AI, apical signal is localized into F- UVJ surface
 717 epithelium and SST cells (inset). (C) While WGA staining is restricted to apical part of F+
 718 UVJ surface epithelial cells, an intense staining in the supranuclear part of SST cells is

719 observed in hens not subjected to AI (inset). (D) After AI, intracellular staining is detected in
 720 UVJ surface epithelial cells, whereas an intense apical (inset i) and luminal staining (inset ii)
 721 are observed inside SST. Figure is representative of several observations. l, lumen; SST,
 722 Sperm Storage Tubule. Scale bar: A-B-C-D, 25 μ m; insets in: A-B-C-D, 25 μ m.

723 **Figure 9.** Staining of GalNAc containing glycans by ECL lectin in the UVJ tissue of F- (A,B)
 724 and F+ (C,D) hens having not been subjected (A,C) or subjected to AI (24h: B,D). (A)
 725 Intracellular staining was limited to some cells of the F- UVJ surface epithelium (inset i),
 726 whereas apical staining was observed in SSTs (inset ii) without AI. (B) After AI, the top of
 727 UVJ epithelial cilia were stained (arrowheads). Supranuclear staining of some SST cells is
 728 observed (encircled). Inset demonstrates the apical (iii) and luminal (iv) staining of SST. (C)
 729 Apical staining on surface epithelium and SST cells is observed. (D) Intracellular staining is
 730 visualized in SSTs (dot circle), and UVJ surface epithelium (inset vi). Inset v focuses on
 731 apical signal in SST cells. Figure is representative of several observations. l, Lumen; SST,
 732 Sperm Storage Tubule. Scale bar: A-B-C-D, 25 μ m; insets in: A-B-C-D, 25 μ m.

733 **Figure 10.** Staining of GalNAc terminating glycans by SBA lectin in the UVJ tissue of F-
 734 (A,B) and F+ (C,D) hens having not been subjected (A,C) or subjected to AI (24h: B,D). (A)
 735 Staining was observed in some F- SST cells of the UVJ (encircled) before AI. Inset shows
 736 apical staining of SST cells. (B) After AI, intense signals are observed on F- SST apices as
 737 well as on the top of UVJ surface epithelial cilia (arrowhead). Insets demonstrate the UVJ
 738 surface epithelial cilia staining (inset i, arrowheads) and luminal staining of SST (inset ii). (C)
 739 Before AI, supranuclear signal is visualized in F+ SST cells (encircled). Inset focuses on the
 740 supranuclear staining in a SST transversal section. (D) After AI, apical and luminal staining is
 741 observed in F+ SST containing resident sperm. Inset shows the intense staining of the top of
 742 UVJ cilia (arrowhead). Figure is representative of several observations. l, Lumen; SST, Sperm
 743 Storage Tubule; SPZ, spermatozoa. Scale bar: A-B-C-D, 25 μ m; insets in: A-B-C-D, 25 μ m.

Without AI

F- OCX36

After AI

Without AI

F+ OCX36

After AI

Comment citer ce document :

Riou, C., Cordeiro, L.-A., Gérard, N. (2017). Eggshell matrix proteins OC-116, OC-17 and OCX36 in hen's sperm storage tubules. *Animal Reproduction Science*, 185, 28-41.

CTL

WGA

SBA

ECL

Vagina

Uterus

Comment citer ce document :

Riou, C., Cordeiro, L.-A., Gérard, N. (2017). Eggshell matrix proteins OC-116, OC-17 and OCX36 in hen's sperm storage tubules. *Animal Reproduction Science*, 185, 28-41.

F- SBA

Without AI

After AI

F+ SBA

Without AI

After AI

Table 1. Lectin conjugates grouped by preferred binding carbohydrate moiety, their sugar specificities and the inhibitory sugars used in control experiments.

Lectin abbreviation	Source of lectin	Concentration ($\mu\text{g/mL}$)	Labelling (min)	time	Sugar specificity	Inhibitory sugar
Galactose/ N-acetylgalactosamine binding lectins						
ECL	Erythrina cristagalli	1.25	60		Gal β 4GlcNAc	Lactose
SBA	Glycine max	1.25	60		Terminal α > Gal	β GalNAc, GalNAc
N-acetylglucosamine lectin						
WGA	Triticum vulgaris	1.25	60		Terminal/internal β GlcNAc >> NeuNAc	GlcNAc with acid or salt

Gal, D-galactose; GalNAc, N-acetylgalactosamine; Glc, D-glucose; GlcNAc, N-acetylglucosamine; NeuNAc, N-acetylneuraminic acid (sialic acid).

Table 2. Comparison of fertility, laying and embryo mortality rates, and efficient (De) and maximum (Dm) duration of fertility between two lines of hens F+ (n=28) and F- (n=27) at 45 weeks of age. Data are expressed as mean $\pm$ SEM. The differences between mean values of the two lines were evaluated using a Mann-Whitney test.

Trait	F+	F-	Level of significance
Fertility rate (%)	58.9 $\pm$ 3.71	31.1 $\pm$ 3.20	P < 0.001
Laying rate (%)	72.2 $\pm$ 4.21	57.4 $\pm$ 3.98	P < 0.01
Embryo mortality of fertile eggs (%)	6.9 $\pm$ 1.65	9.6 $\pm$ 1.72	NS
Efficient duration of fertility (days)	3.6 $\pm$ 0.69	1.7 $\pm$ 0.23	P < 0.05
Maximum duration of fertility (days)	13.3 $\pm$ 0.82	7.3 $\pm$ 0.75	P < 0.001

Declaration of interest

The authors declare no conflict of interest.