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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

## 30 1. Introduction

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

42 The SST epithelium is structured in two distinct regions that are connected to each other.  
43 The upper portion which opens into the uterovaginal lumen composed of ciliated cells, and  
44 the 'blind portion' exclusively made-up of non-ciliated cells (Van Krey et al., 1967) (Burke et  
45 al., 1972; Tingari and Lake, 1973; Bakst and Bauchan, 2015; Waqas et al., 2016). Non-  
46 ciliated SST cells are the functional portion dedicated to sperm storage in the SST epithelium.  
47 These secretory cells were reported to release apocrinal secretions and exosomes (Bakst and  
48 Bauchan, 2015; Waqas et al., 2016). They were also reported to contain glycogen, lipids,  
49 phospholipid, acid phosphatase (Gilbert et al., 1968; Huang et al., 2016), calcium (Holm et  
50 al., 2000) and carbonic anhydrase (Holm et al., 1996). Some of these products, like calcium or  
51 carbonic anhydrase, were assumed to impact sperm survival (Fujihara and Koga, 1984; Holm  
52 and Ridderstrale, 1998; Holm and Wishart, 1998; Holm et al., 2000). The SST lumen is filled  
53 with fluid that also contains several essential factors such as acid phosphatase, lipids (Gilbert  
54 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<sub>3</sub> 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  $200 \times 10^6$  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

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

130 Spermatozoa from 6 broiler breeder males were rinsed 3 times in BPSE diluent (10 min,  
131 600g). They were incubated at 41°C during 1 hour in UF collected from F- or F+ hens at a  
132 final concentration of  $1 \times 10^6$  sperm/mL. Spermatozoa were rinsed in PBS and fixed in 4%  
133 saline buffered (PBS) formalin before immunohistochemistry procedure.

#### 134 2.4. Tissue Collection

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

#### 141 2.5. Western blotting

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

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

#### 161 2.6. *Histochemistry Preparation for Immuno and Lectin Detection*

162 Following 24 hours fixation in 4% saline buffered (PBS) formalin, tissue samples (uterus,  
163 vagina and UVJ) were transferred to two successive bathes of 70% ethanol (2x30 min), and  
164 then moved to an automated system tissue processor (Leica TP1020 Semi-enclosed Benchtop  
165 Tissue Processor). The samples were then embedded in paraffin (Leica EG1150 Modular  
166 Tissue Embedding Center, Leica Microsystems Richmond, Inc., Richmond, IL). Four to six 7-  
167 µm thick sections were collected in sequence onto slides treated with albumin, air-dried  
168 overnight, stored at room temperature (RT) for 12 hours and then incubated overnight at  
169 60°C. The staining procedures consisted in deparaffinization for 5 min in toluene, followed by  
170 progressive rehydration (100%, 95%, 80% and 70% ethanol bathes, 2 min each) and removal  
171 of ethanol excess by water bathes.

#### 172 2.7. *Immunohistochemistry*

173 After dehydration, the slides were treated with 1% citrate based unmasking solution H3300  
174 (Vector Laboratories, Burlingame, CA) diluted as recommended by the manufacturer. The  
175 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  $P <$

225 0.001). Moreover, the laying rates were significantly higher in F+ hens than in F- hens ( $P <$   
226 0.01) over the experimental period. No significant difference between the two lines was  
227 recorded in the percentage of embryo survival, irrespective of the week following  
228 insemination.

### 229 3.2. *OC-116, OC-17 and OCX36 in uterine fluid (UF)*

230 OC-116, OC-17 and OCX36 were detected by Western blot in UF at 116kDa, 36kDa and  
231 17kDa, respectively (Figure 2A). As shown in Figure 2B, the signals were stronger for F+  
232 hens than for F- hens, even though the difference is not significant. Nevertheless, the  
233 immuno-detection signals of all the three proteins tended to increase after AI in F- hens,  
234 whereas they tended to decrease in F+ hens.

### 235 3.3. *OC-116, OC-17 and OCX36 immunohistochemistry in vagina and uterus*

236 We observed that the epithelium of the vagina and uterus showed different affinities for  
237 OC-116, OC-17 and OCX36 antibodies. None of the 3 proteins were detected in the vagina  
238 epithelium (Figures 3A-C). OC-116 was localized in the uterine surface epithelium (Figure  
239 3E) while OC-17 was localized exclusively in the uterine glandular epithelium (Figure 3F).  
240 OCX36 was detected neither in the uterine epithelium, nor in the uterine glandular epithelium  
241 (Figure 3G). Negative controls for immunohistochemistry demonstrated the absence of  
242 unspecific signal in vagina (Figure 3D) and uterus (Figure 3H).

### 243 3.4. *OC-116, OC-17 and OCX36 immunohistochemistry in UVJ*

#### 244 3.4.1. *OC-116 protein*

245 OC-116 was localized in UVJ surface epithelial cells and in SST cells cytoplasm (Figures  
246 4A-D). Moreover, in both F+ and F- hens, the signal appeared luminal even before AI  
247 (Figures 4A, 4C, 4C inset) or after AI (Figures 4B, 4B inset i, 4D, 4D insets iii and v).  
248 Interestingly after AI, the luminal staining was in contact with spermatozoa in F- hens (Figure

249 4B inset ii) and surrounded spermatozoa in F+ hens (Figure 4D insets v and vi). Moreover, we  
250 observed a granular secretory product with an intense staining for OC-116 projecting outside  
251 SST (Figure 4D insets iii and iv). OC-116 displayed a crown-shaped signal around spherical  
252 structures localized inside SST (Figure 4D inset vii).

#### 253 3.4.2. *OC-17 protein*

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

#### 264 3.4.3. *OCX36 protein*

265 In UVJ, no signal for OCX36 was detected in surface epithelial cells, but was specifically  
266 observed in SST cells from both F+ and F- hens, either without AI or 24h after AI (Figures  
267 6A-D). Without AI, the signal was heterogeneous, and located in the cytoplasm of SST cells  
268 in both F- (Figure 6A) and F+ (Figure 6C) hens. Moreover, a granular signal was also  
269 observed projecting in the lumen of SST from F- (Figure 6A insets i and ii) and F+ hens  
270 (Figure 6C inset). After AI, the localization of OCX36 was mainly luminal in F- hens, with  
271 only a small amount observed in the cytoplasm of few cells (Figure 6B). Some spermatozoa  
272 appeared enclosed in a substance positive for OCX36 staining (Figure 6B inset). In contrast,

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

### 275 3.5. *Lectin histochemistry*

#### 276 3.5.1. *Lactosamine (Gal $\beta$ 1- $\rightarrow$ 4GlcNAc), Terminal N-acetylgalactosamine (GalNAc) and* 277 *N-acetylglucosamine (GlcNAc) residues in the vagina and uterus*

278 As shown in Figure 7, we observed that in vaginal and uterine tissues, ECL and WGA  
279 bound with high affinity the Gal $\beta$ 1- $\rightarrow$ 4GlcNAc and the GlcNAc residues, respectively.  
280 Moreover, SBA bound with low affinity the GalNAc residues from both tissues. In vagina,  
281 ECL staining was strong in the cytoplasm of some epithelial cells, as well as in loose  
282 connective tissue (Figure 7A). Moreover, SBA lectin bound to lymphocytes of loose  
283 connective tissue (Figure 7B), and WGA bound with strong affinity to the cytoplasmic part of  
284 some epithelial cells (Figure 7C). Negative controls for lectin histochemistry demonstrated  
285 the absence of unspecific signal in vagina (Figure 7D). In uterus, ECL staining was  
286 demonstrated in the cytoplasmic and the apical part of surface epithelial cells (Figure 7E), and  
287 of tubular gland cells (Figure 7E inset, arrowhead). SBA lectin bound to lymphocytes of loose  
288 connective tissue, as previously observed in the vagina (Figure 7F). WGA strongly bound to  
289 the apical part of uterine surface epithelial cells as well as that of tubular gland cells (Figure  
290 7G inset, arrowhead), and with less intensity to their cytoplasmic compartment (Figure 7G).  
291 Negative controls for lectin histochemistry demonstrated the absence of unspecific signal in  
292 uterus (Figure 7H).

#### 293 3.5.2. *N-acetylglucosamine (GlcNAc) residues in UVJ*

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

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

### 307 3.5.3. *Lactosamine (Gal $\beta$ 1- $\rightarrow$ 4GlcNAc) residues in the UVJ*

308 In UVJ, ECL binding differed between F+ and F- hens, both in non-inseminated and  
309 inseminated females (Figure 9). In non-inseminated F-, it appeared bound irregularly and  
310 strongly to cytoplasmic and apical parts of some surface epithelial cells and SST cells (Figure  
311 9 A inset i and ii). In non-inseminated F+ hens, it appeared bound regularly to the apical part  
312 of epithelial and SST cells (Figure 9C). Moreover, ECL staining was visualized in loose  
313 connective tissue from F- UVJ tissue, but not in that from F+. After AI, ECL displayed  
314 specific staining patterns. In F- UVJ tissue, it homogeneously bound to the top of cilia from  
315 the surface epithelium (Figure 9B, arrowhead), as well as to the apical and the cytoplasmic  
316 parts of SST cells (Figure 9B inset iii). Moreover, some SST cells showed an intense ECL  
317 staining. We also observed that the SST lumen displayed ECL staining (Figure 9B inset iv).  
318 Furthermore, the F- UVJ loose and dense connective tissue was strongly stained by ECL  
319 lectin. In F+ hens after AI, ECL staining was observed irregular and strong in cytoplasmic  
320 and apical parts of some surface epithelial cells (Figure 9D inset vi) and some SST cells

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

#### 323 3.5.4. Terminal *N*-acetylgalactosamine (*GalNAc*) residues in the UVJ

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

333

## 334 4. Discussion

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

339 Using two lines of hens (F+ and F-), we showed that the genetic divergence on the duration  
340 of fertility remains as originally described (Brillard et al., 1998). The F- hens are  
341 characterized by a reduced fertility, a reduced laying rates, as well as shorter efficient and  
342 maximal durations of fertility when compared to the F+ line. Fertilization rates after the first,  
343 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 (TGF $\beta$ ), 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

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

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

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

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

443 hen's oviduct (Nakano et al., 2002). Moreover, dermatan sulfate proteoglycan OC-116 is a  
444 galactosaminoglycan composed of N-acetylgalactosamine and N-acetylglucosamine residues  
445 that combine to form the major motif that is present on the molecule, LacDiNAc (GalNAc $\beta$ 1-  
446 4GlcNAc). Interestingly, OC-116 contains several fucosylated forms of LacDiNAc motif  
447 (Nimtz et al., 2004). However, fucose residues are known to be key components of sperm  
448 binding to mammalian oviduct (Suarez, 2008). Furthermore, it has been reported that OC-17  
449 contains N-acetylgalactosamine, but not N-acetylglucosamine moieties (Hincke et al., 1995).  
450 Therefore, a better understanding of the role of the eggshell matrix proteins (OC-116 and OC-  
451 17) and their N-acetylgalactosamine and N-acetylglucosamine moieties is needed in the  
452 context of sperm adherence on SST epithelial cells.

453 In conclusion, this is the first study reporting the presence of OC-116, OCX36 and OC-17  
454 eggshell matrix proteins in SSTs, their concomitant expression with Gal/GalNAc and  
455 Glc/GlcNAc, as well as with calcium and sperm. Our results suggest that the storage of sperm  
456 in the SSTs and the eggshell mineralization process, assumed by OC-116, OCX36 and OC-  
457 17, may be related. In our view, the present study also opens up new research prospects. Thus,  
458 the study of the presence of eggshell matrix proteins, glycoconjugates and calcium in  
459 infundibulum, specifically in SSTs, should receive interest to better understand their role in  
460 avian species like in F<sup>+</sup> and F<sup>-</sup> hen genetic lines. Another matter of interest refers to the main  
461 biochemical properties of the eggshell matrix proteins which appear well conserved in most  
462 avian species and other sauropsids (snakes, crocodiles, turtles) (Panheleux et al., 1999; Bardet  
463 et al., 2010). Considering that these species are also known to maintain sperm survival in their  
464 genital tract (Holt, 2011), the study of eggshell matrix proteins expression should receive  
465 additional attention to better understand their role(s) at maintaining a local environment  
466 favoring the long term storage of sperm in SSTs.

467

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644

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  $200 \times 10^6$  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  $\mu$ 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  $\mu$ m; insets  
 676 in: B-C-D.iv-D.v-D.vi, 10  $\mu$ m, insets in: D.iii-D.vii, 20  $\mu$ 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  $\mu$ m; insets in: B-D, 20  $\mu$ 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  $\mu$ m; insets in: A-D, 10  $\mu$ m.

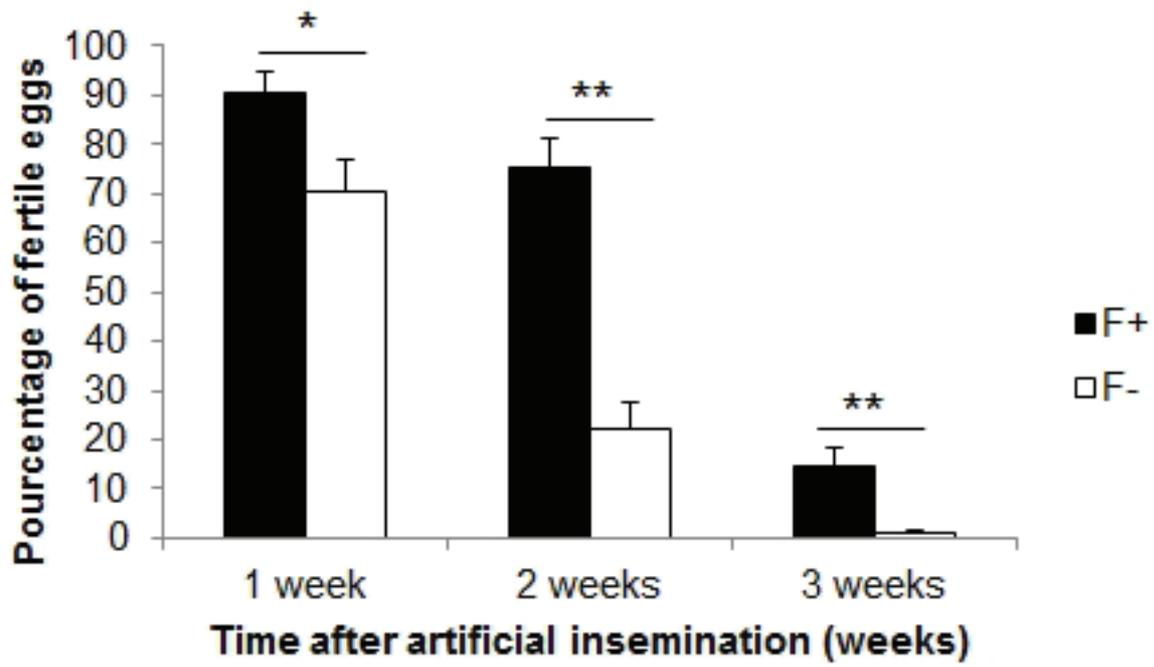
700 **Figure 7.** Staining of vagina (A,B,C,D) and uterus (E,F,G,H) tissue incubated with ECL  
 701 (A,D), SBA (B,E) and WGA (C,F) lectins. (A) Mucosa from vagina demonstrates ECL  
 702 positive reactivity. Inset shows the granular aspect of the ECL staining in some epithelial cells  
 703 of vaginal 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  $\mu$ m; insets in: A-C-E-G, 10  $\mu$ m.

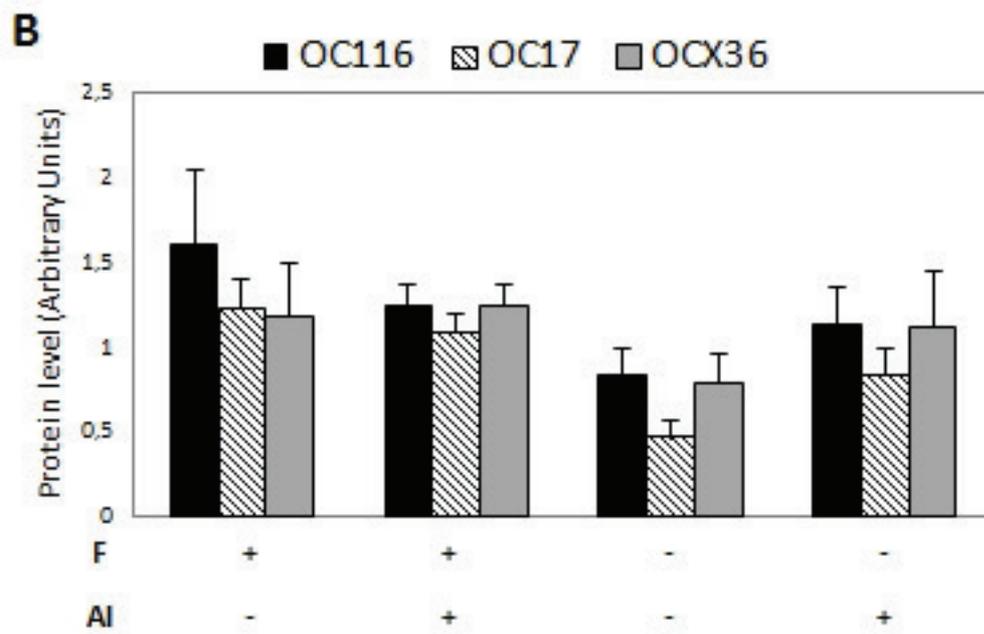
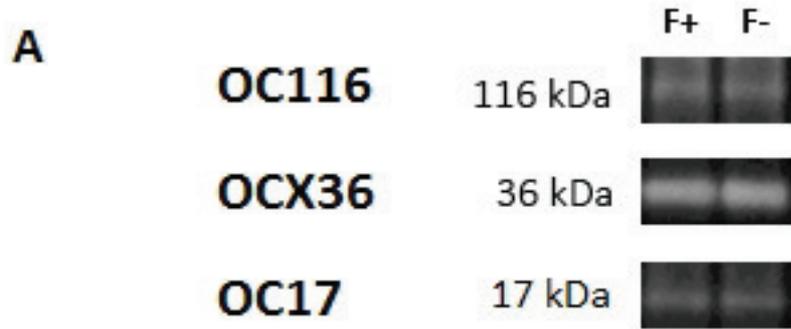
713 **Figure 8.** Staining of GlcNAc containing glycans by WGA lectin in the UVJ tissue of F-  
 714 (A,B) and F+ (C,D) hens having not been subjected (A,C) or subjected to AI (24h: B,D). (A)  
 715 Intracellular staining is detected in the F- UVJ surface epithelial cells as well as in the  
 716 transition 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  $\mu$ m; insets in: A-B-C-D, 25  $\mu$ 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  $\mu$ m; insets in: A-B-C-D, 25  $\mu$ 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  $\mu$ m; insets in: A-B-C-D, 25  $\mu$ m.

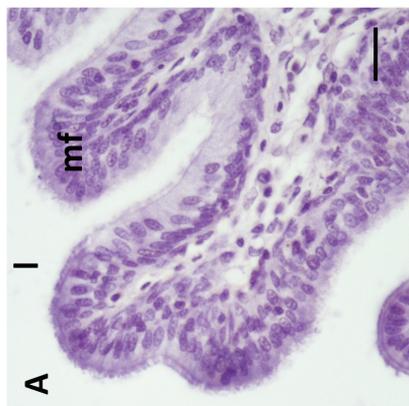




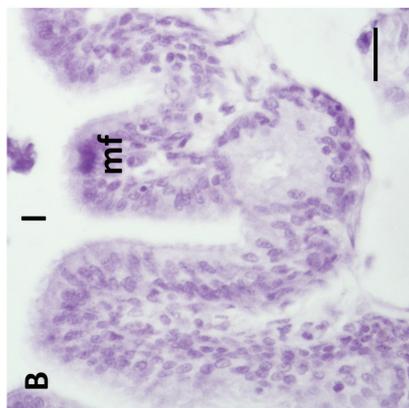
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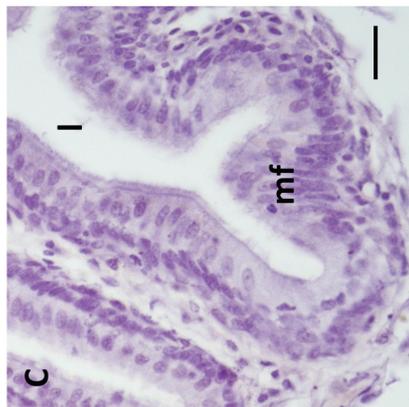
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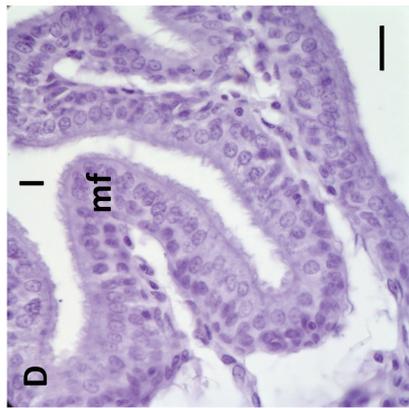
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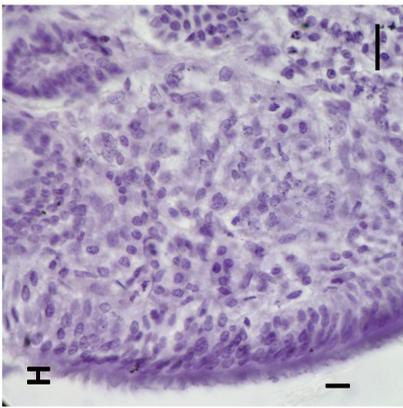
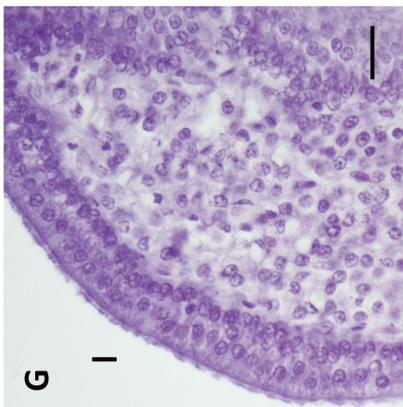
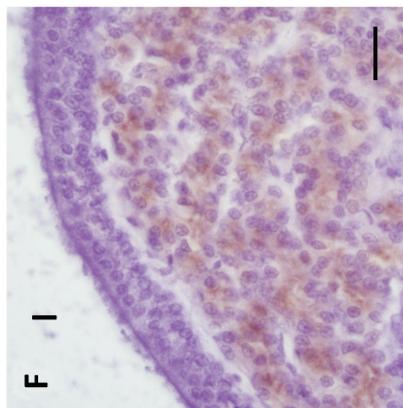
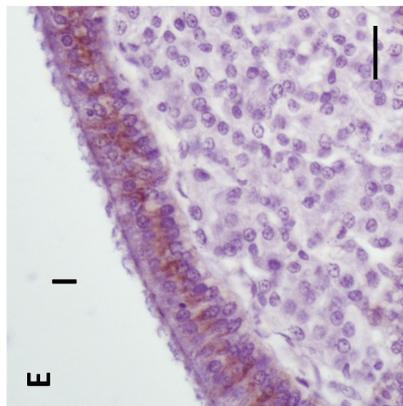
OCX36



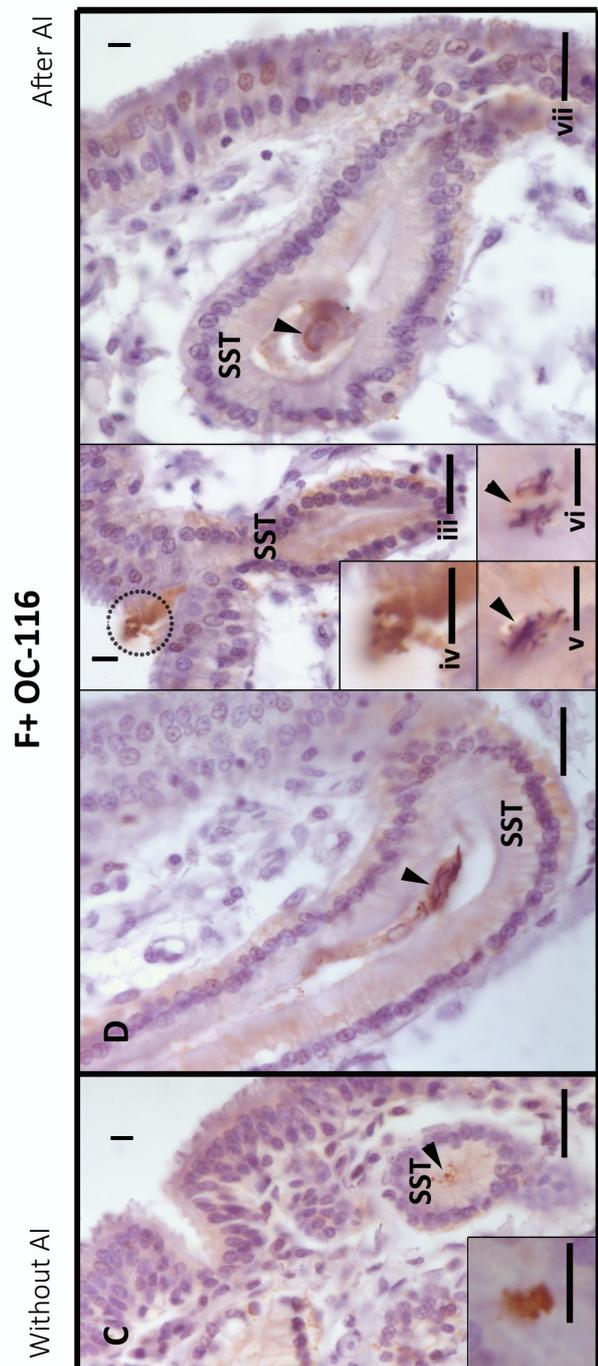
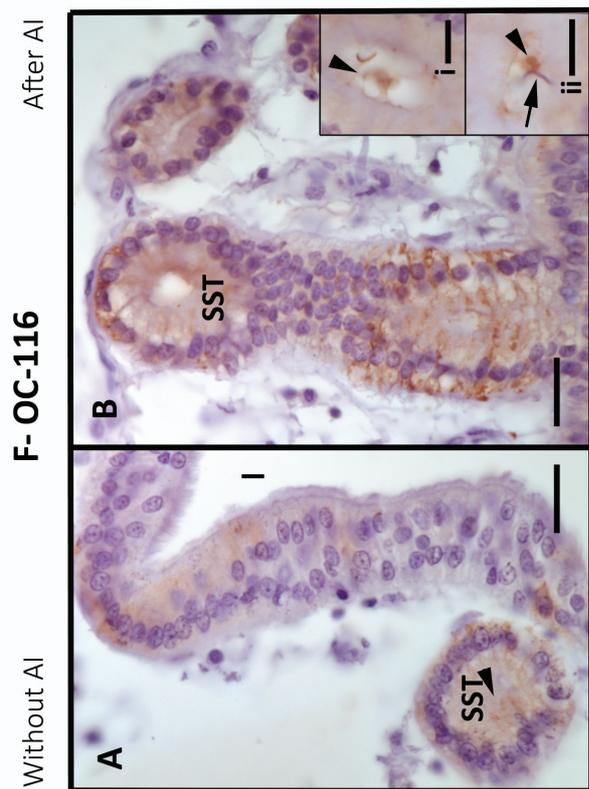
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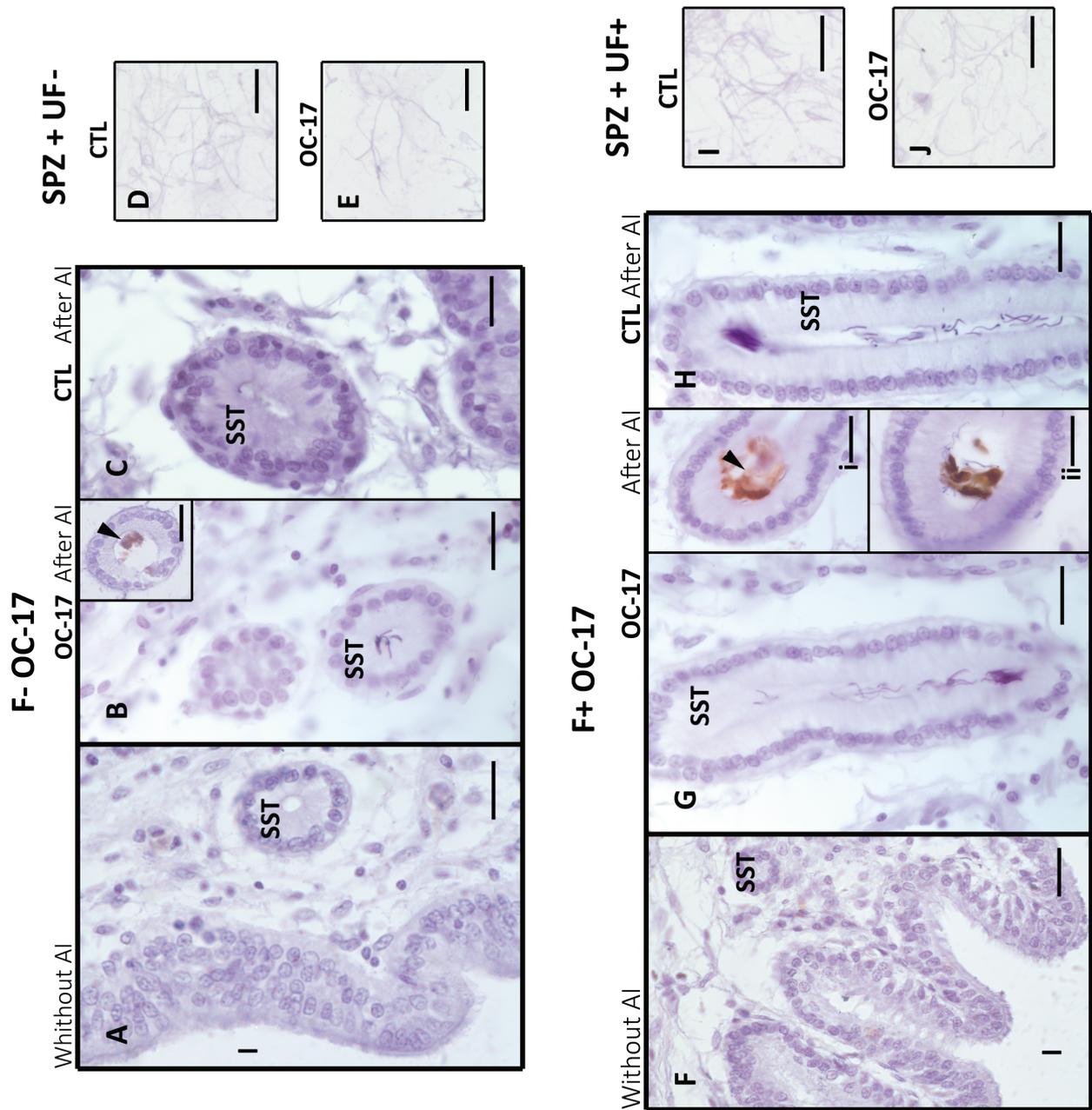


Vagina



Uterus





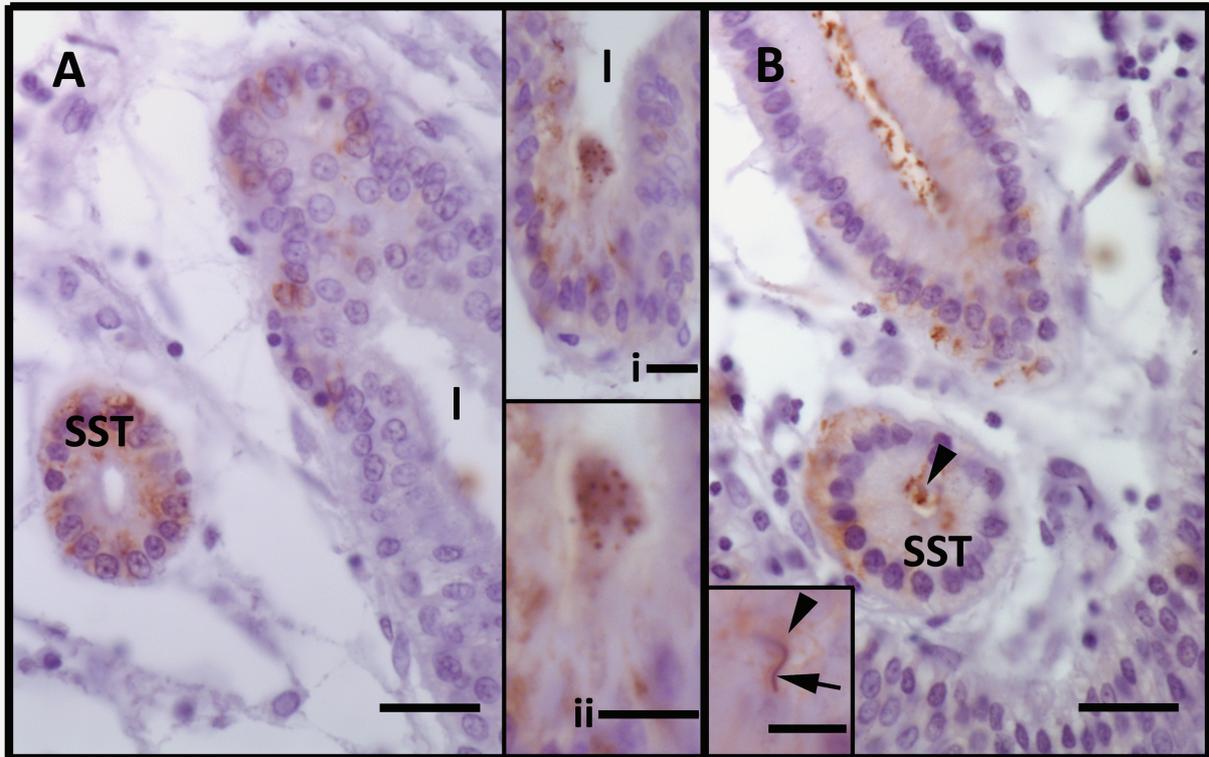
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Without AI

### F- OCX36

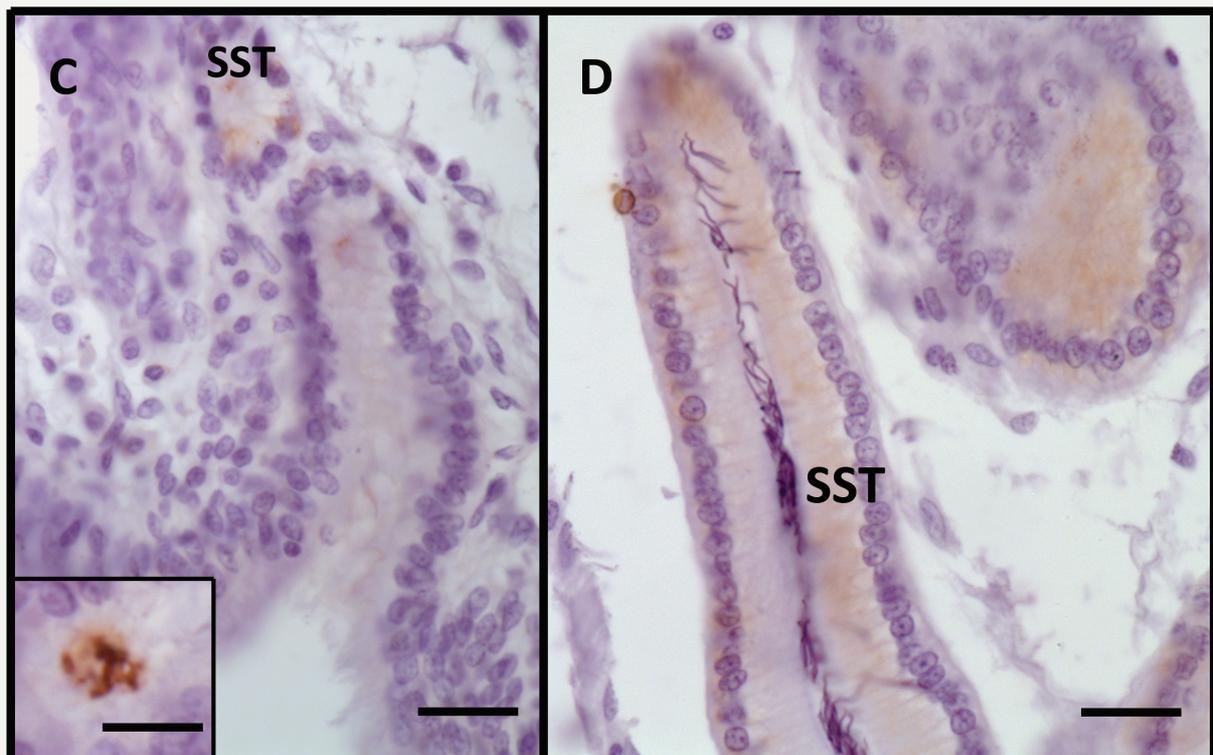
After AI



Without AI

### F+ OCX36

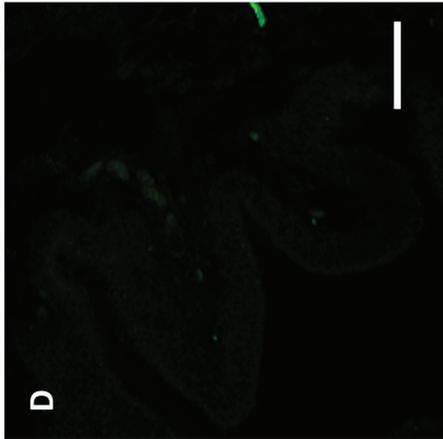
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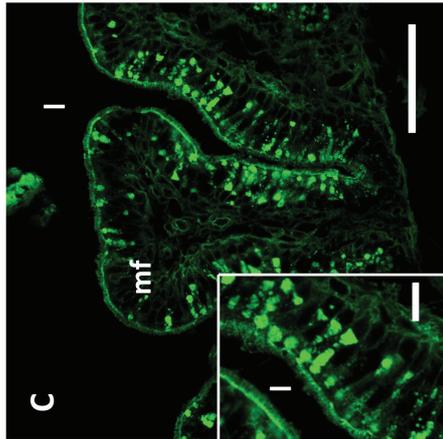
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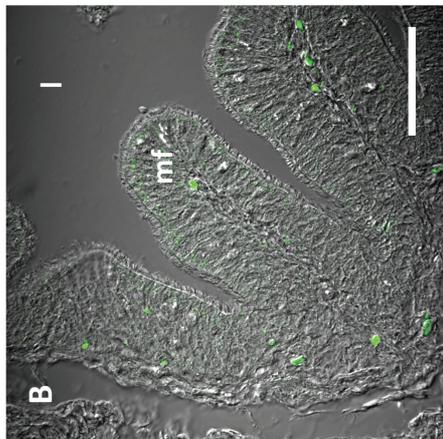
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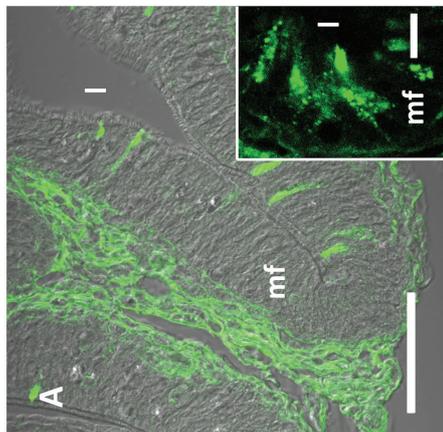
WGA



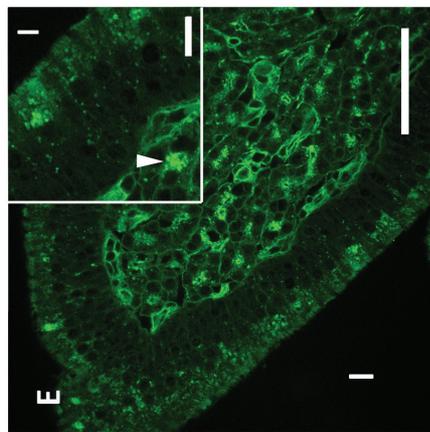
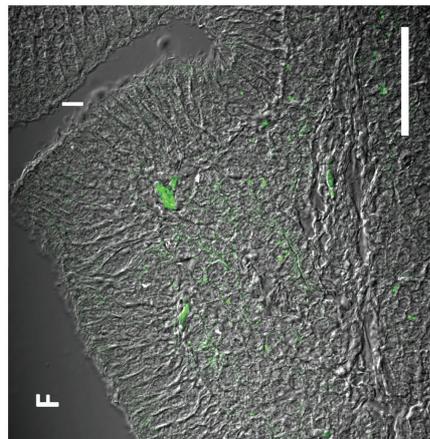
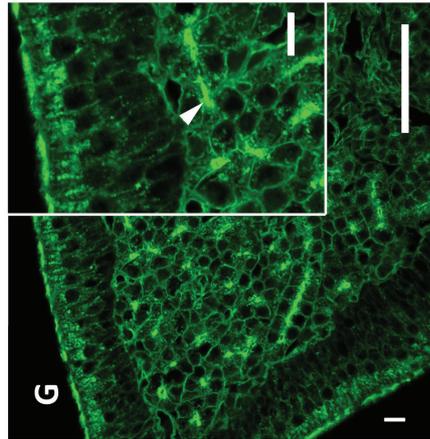
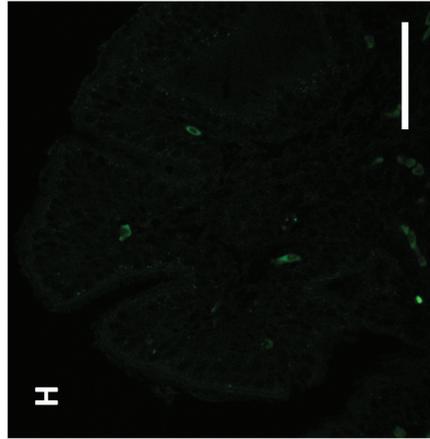
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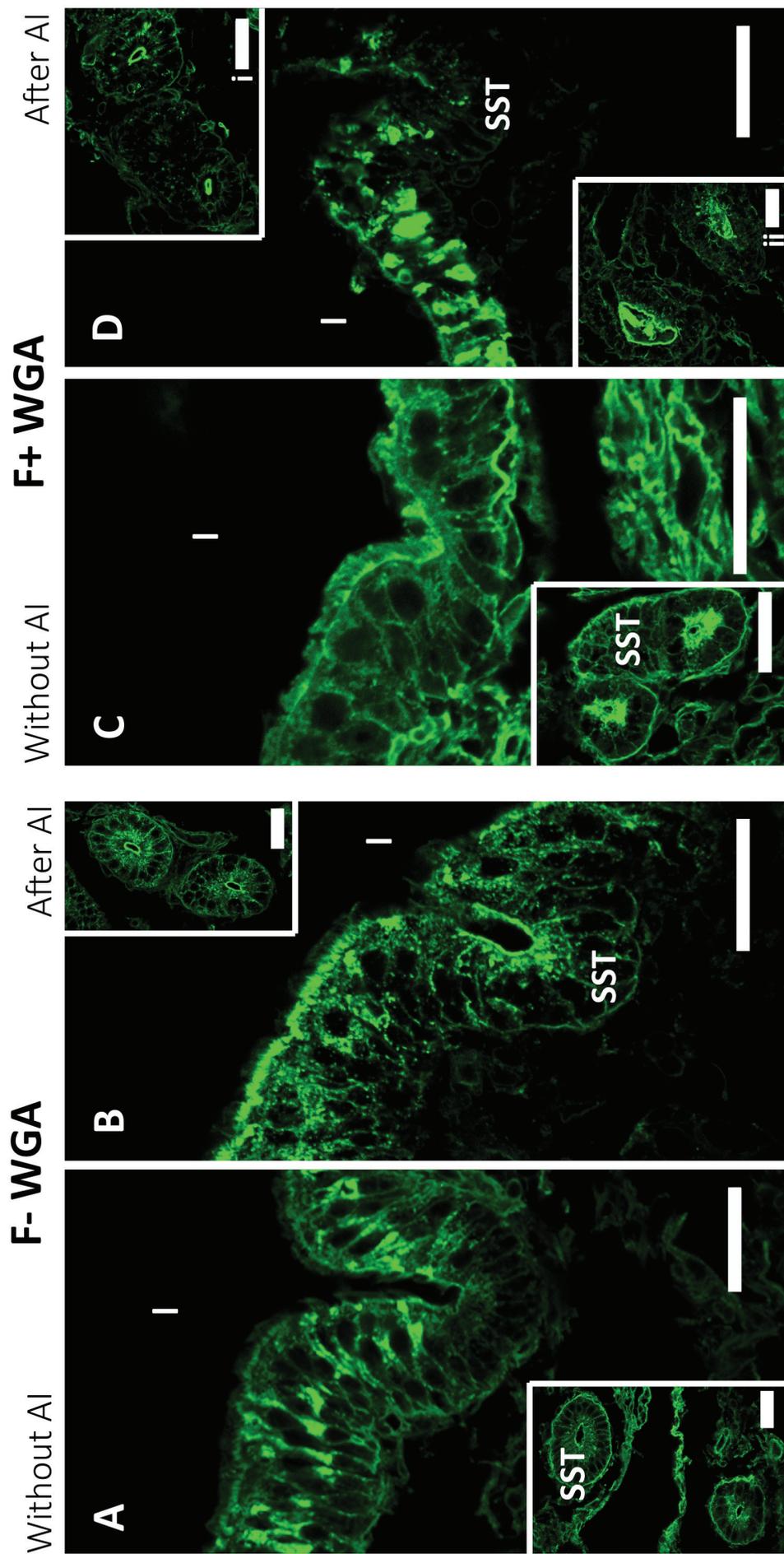
ECL



Vagina

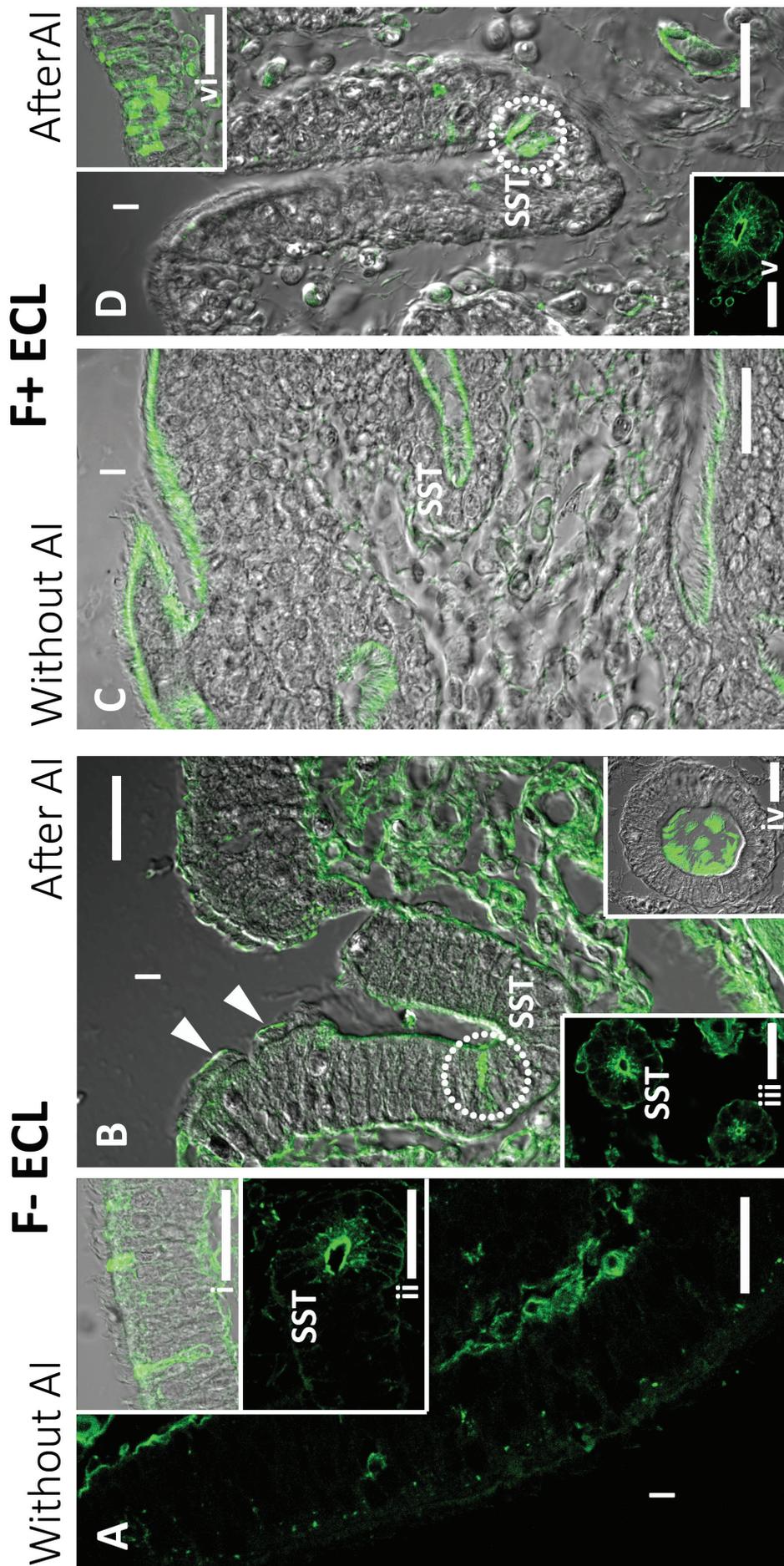


Uterus



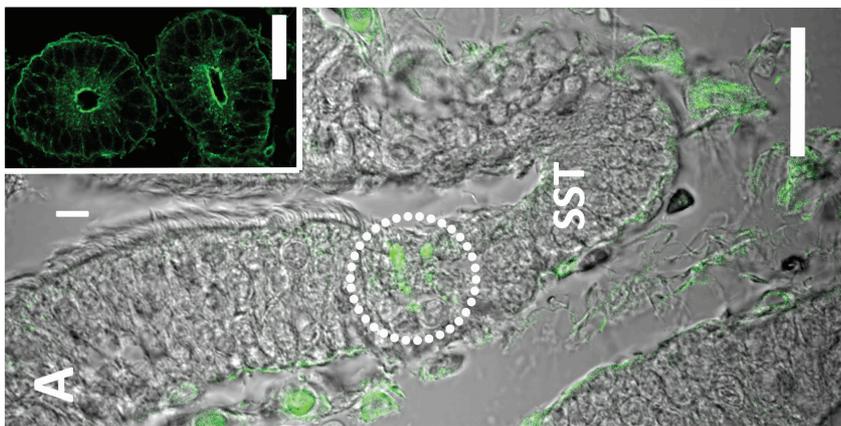
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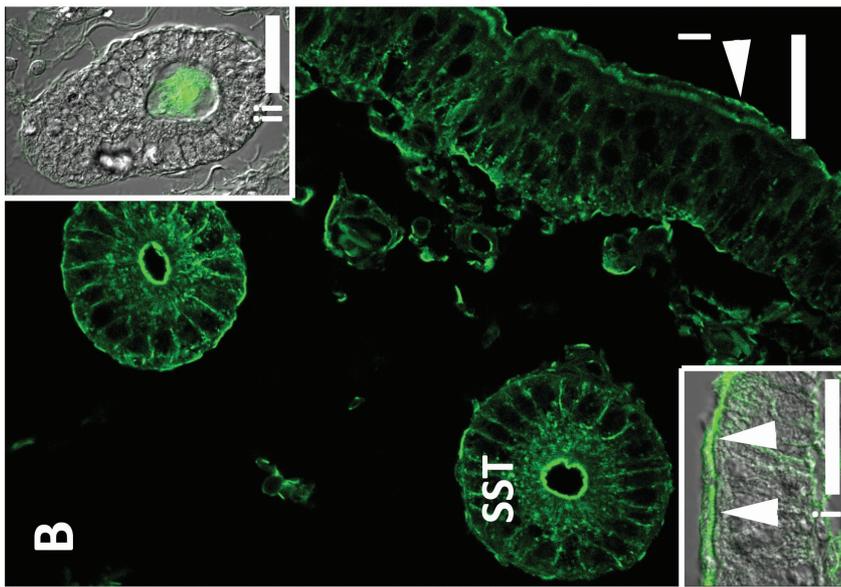


**F- SBA**

Without AI

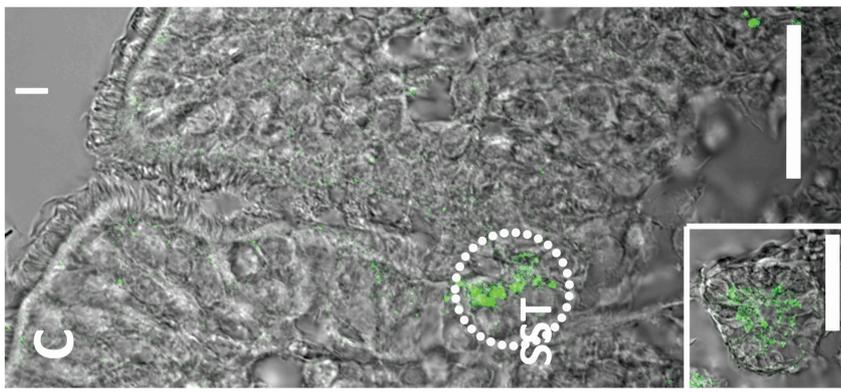


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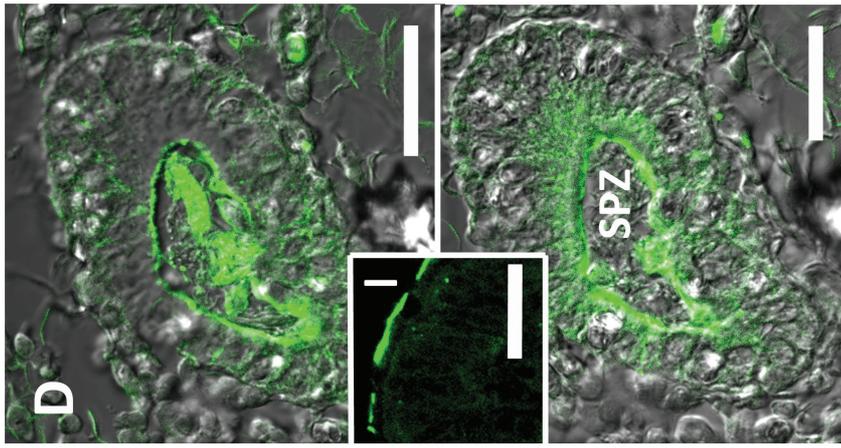


**F+ SBA**

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
<b>Galactose/ N-acetylgalactosamine binding lectins</b>						
ECL	Erythrina cristagalli	1.25	60		Gal $\beta$ 4GlcNAc	Lactose
SBA	Glycine max	1.25	60		Terminal $\alpha$ >	$\beta$ GalNAc, GalNAc
<b>N-acetylglucosamine lectin</b>						
WGA	Triticum vulgaris	1.25	60		Terminal/internal  $\beta$ 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.

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

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## **Declaration of interest**

The authors declare no conflict of interest.