

1 running head: Peláez and Long, Poultry sperm glycocalyx characterization

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3 Characterizing the Glycocalyx of Poultry Spermatozoa: I. Identification and Distribution of  
4 Carbohydrate Residues using Flow Cytometry and Epifluorescence Microscopy

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**Abstract**

The aim of the present work was to use a battery of lectins to (1) delineate the carbohydrate content of sperm glycocalyx in the turkey and chicken using flow cytometry analysis and (2) evaluate the distribution of existing sugars over the sperm plasma membrane surface with epifluorescent microscopy. Carbohydrate groups (corresponding lectins) that were investigated included galactose (GS-I, Jacalin, RCA-I, PNA), glucose and/or mannose (Con A, PSA, GNA), N-acetyl-glucosamine (GS-II, s-WGA, STA), N-acetyl-galactosamine (SBA, WFA), fucose (Lotus, UEA-I), sialic acid (LFA, LPA) and N-acetyl-lactosamine (ECA). Spermatozoa were assessed before and after treatment with neuraminidase to remove sialic acid. Mean Fluorescence Intensity (MnFI) was used as indicator of lectin binding for flow cytometry analysis. Non-treated spermatozoa from both species showed high MnFI when incubated with RCA-I, Con A, LFA and LPA, as did chicken spermatozoa incubated with s-WGA. Neuraminidase treatment increased the MnFI for most lectins except LFA and LPA, as expected. Differences in MnFI between species included higher values for s-WGA and ECA in chicken spermatozoa and for WFA in turkey spermatozoa. Microscopy revealed segregation of some sugar residues into membrane-specific domains; however, the two staining techniques (cell suspension versus fixed preparation) differed in identifying lectin binding patterns, with fixed preparations yielding a high degree of non-specific binding. We conclude that (1) the glycocalyx of turkey and chicken spermatozoa contains a diversity of carbohydrate groups, (2) these residues are extensively masked by sialic acid, (3) the glycocalyx composition is species-specific, and (4) some glycoconjugates appear to be segregated into membrane-specific domains. Characterization of the poultry sperm glycocalyx is the first step in identifying the physiological impact of semen storage on sperm function.

43 Key words: turkey; chicken; semen; glycoconjugate; lectin

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## 44           **Introduction**

45           The surface of all eucaryotic cells consists of a carbohydrate-rich zone known as the  
46 glycoalyx. This cell coat contains oligosaccharide chains covalently bound to integral plasma  
47 membrane proteins (glycoproteins) or lipids (glycolipids), as well as polysaccharide chains  
48 covalently linked to a protein core (proteoglycan) that either extends across or is attached to the  
49 lipid bilayer. Glycoproteins and proteoglycans adsorbed onto the cell surface also contribute to  
50 the glycoalyx of many types of cells (Alberts et al, 1994). The glycoalyx of a typical somatic  
51 cell, such as the human erythrocyte, is about 10 nm in thickness (Rademacher et al, 1988). In  
52 general, the glycoalyx functions to mediate specific recognition events and/or provide  
53 modulation of biological processes (Varki, 1993).

54           Similar to somatic cells, the glycoalyx of spermatozoa is critical for many specific cell  
55 functions. A notable difference between the glycoalyx of somatic cells and spermatozoa is the  
56 depth of the carbohydrate layer, with maximum thickness of 60 nm reported for guinea pig  
57 spermatozoa (Bearer and Friend, 1990). Moreover, the sperm cell glycoalyx appears to be  
58 extremely complex in both composition and organization compared to other cell types. For  
59 example, the glycoalyx of mammalian oocytes contains three or four different families of  
60 glycoproteins (Yanagimachi, 1988), whereas that of human spermatozoa is estimated to contain  
61 between 50 and 150 different glycoconjugates, which are segregated into different functional  
62 domains rather than being homogeneously distributed on the cell surface (Schröter et al, 1999).  
63 The composition of the mammalian sperm glycoalyx also is dynamic, with the original  
64 carbohydrate layer being extensively modified during the transit through the epididymis and the  
65 female reproductive tract (Eddy, 1988). The sperm glycoalyx represents the primary interface  
66 between the male gamete and its environment and, although specific functions have not been

67 completely determined, is known to be involved with immunoprotection in the female genital  
68 tract, acquisition of fertilizing ability, acrosome reaction, and early gamete interactions (Schröter  
69 et al, 1999; Diekman, 2003).

70 Most of the data for sperm glycocalyx pertaining to composition or functionality has been  
71 derived from mammalian species. A limited number of studies have been conducted with other  
72 taxa, such as marsupials (Cooper et al, 2001), invertebrates (Perotti and Pasini, 1995) and fishes  
73 (Rojas and Esponda, 2001) documenting the composition, distribution and/or functional  
74 implications of surface membrane glycoconjugates. In contrast, few data regarding the  
75 composition, spatial distribution or function of the avian sperm cell glycocalyx have been  
76 reported. It is known that the chicken sperm glycocalyx contains residues of sialic acid (Froman  
77 and Thurston, 1984), as well as  $\alpha$ -glucose and/or  $\alpha$ -mannose (Bakst and Howarth, 1977). Based  
78 on what has been reported for other species, however, the avian sperm glycocalyx should contain  
79 a complex diversity of carbohydrates that are critical for sperm function. Currently, the only  
80 functions associated with the avian sperm glycocalyx are that terminal sialic acid residues are  
81 necessary for chicken spermatozoa to traverse the vagina and become sequestered within the  
82 sperm storage tubules in the hen's reproductive tract (Froman and Engel, 1989). Before specific  
83 poultry sperm cell functions can be attributed to the glycocalyx, the carbohydrate content and  
84 distribution needs to be delineated. Accordingly, our objective here was to characterize the types  
85 of sugar residues comprising the glycocalyx of poultry spermatozoa. Specifically, a battery of  
86 FITC-labeled lectins were used in combination with flow cytometry and epifluorescent  
87 microscopy to determine the types and distribution of carbohydrate residues present on the  
88 plasma membrane of both turkey and chicken spermatozoa.

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90           **Methods**

91           Animals.

92           Male turkeys<sup>1</sup> and roosters<sup>2</sup> used in the study were maintained using standard  
93 management practices in the Beltsville Agricultural Research Center poultry facilities under  
94 lighting conditions (14L:10D light cycle, turkey; 16L:8D light cycle, chicken) for sperm  
95 production. Prior to the initiation of the study, males were evaluated for sperm mobility as  
96 described by Froman and McLean (1996) with minor modifications (Long and Kramer, 2003).  
97 Briefly, a suspension of  $1 \times 10^9$  sperm/mL in mobility buffer was overlaid onto a 6% (wt/vol)  
98 Accudenz<sup>3</sup> solution, and the spermatozoa allowed to swim down into the Accudenz for 5 min at  
99 41°C. The optical density of the solution was then measured with a photometer<sup>4</sup> at 540 nm after 1  
100 min of equilibration. It has been demonstrated that the frequency of sperm mobility values for  
101 individual males approximates a normal distribution (Froman and Feltmann, 1998). Nine males  
102 of average mobility (values in the mean  $\pm$  standard deviation range) from each species were  
103 randomly assigned to one of three groups (3 males/group).

104           Semen collection and processing.

105           Semen was collected manually (Burrows and Quinn, 1937) from both turkeys and  
106 roosters on a weekly basis, pooled within the designated group and diluted 1:1 with Beltsville  
107 Poultry Semen Extender II<sup>5</sup>. Seminal plasma was removed using the Accudenz washing method

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<sup>1</sup> Hybrid Grade Maker, Hybrid Turkeys, Ontario, Canada

<sup>2</sup> Hy-Line W-36, Hy-Line International, Elizabethtown, PA

<sup>3</sup> Accudenz, Accurate Chemical & Scientific Corporation, Westbury, NY

<sup>4</sup> IMV Microreader, IMV International Co., Minneapolis, MN

<sup>5</sup> Continental Plastics Corp., Delavan, WI.

108 (McLean et al, 1998). Briefly, diluted semen (500  $\mu$ L, turkey; 750  $\mu$ L, chicken) was gently  
109 layered on top of the discontinuous gradient (12% Accudenz, 5 mL; 30% Accudenz, 0.5 mL) and  
110 centrifuged (1,250 x g; 4°C) for 25 min with a gradual stop (i.e., without using the brake).  
111 Spermatozoa present at the interface between the Accudenz layers were recovered and aliquoted  
112 for immediate lectin staining or neuraminidase treatment followed by lectin staining.

### 113 Lectins, Inhibitory Sugars and Neuraminidase Treatment.

114 Seventeen FITC-conjugated lectins<sup>6</sup> were used to detect residues of 7 carbohydrate  
115 groups: 1) galactose (GS-I, Jacalin, RCA-I, PNA), 2) glucose and/or mannose (Con A, PSA,  
116 GNA), 3) N-acetyl-glucosamine [GS-II, succinyl-WGA (s-WGA), STA], 4) N-acetyl-  
117 galactosamine (SBA, WFA), 5) fucose (Lotus, UEA-I), 6) sialic acid (LFA, LPA) and 7) N-  
118 acetyl-lactosamine (ECA). The specific carbohydrate affinity and inhibitory sugar of each lectin  
119 is shown in Table 1. For all experiments, lectins were used at a concentration of 100  $\mu$ g /mL in  
120 Tris buffer (0.05 M Tris, 0.15 M NaCl; pH 7.6: TBS). GS-I, GS-II, Con A, PSA and UEA-I were  
121 prepared in TBS containing 1 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>. LPA was prepared in TBS  
122 containing 10 mM CaCl<sub>2</sub>, pH 8.0. Sperm processing methods for lectin staining were dependent  
123 upon the type of fluorometric analysis (flow cytometry or microscope) as detailed below  
124 (Experiments 1-3).

125 The specificity of lectin binding was confirmed using competitive inhibition with free  
126 sugars for all lectins except LFA and LPA. Lectins were pre-incubated with specific inhibitory  
127 carbohydrates<sup>7</sup> for at least 1 h prior to incubation with spermatozoa. The molar concentration of  
128 all inhibitory sugars except chitin ranged from 100 to 400 mM, depending upon the success of

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<sup>6</sup> EY Laboratories Inc., San Mateo, CA

<sup>7</sup> Sigma Chemical Co., St. Louis, MO; except Lactose and Fucose (EY Laboratories)

129 lectin inhibition and the method of fluorometric assessment. Because of the polymeric structure  
130 of chitin, the precise molar concentration of this sugar could not be determined. Instead, an iso-  
131 osmotic solution was prepared by dissolving 10  $\mu\text{g}$  of chitin in 1 mL of TBS, and the inhibitory  
132 action of the sugar was evaluated at 3 concentrations (2.5, 5 and 10  $\mu\text{g}/\text{mL}$ ). The specificity of  
133 binding for lectins recognizing sialic acid was confirmed using a neuraminidase treatment to  
134 enzymatically remove terminal sialic acid residues.

135 Terminal sialic acid residues also are known to mask other sugar residues (Cooper et al,  
136 2001); therefore, neuraminidase treatment was used to verify the presence of masked  
137 carbohydrates. Spermatozoa recovered from the Accudenz gradient were resuspended in TBS,  
138 pH 6.0, to a final concentration of  $1 \times 10^9$  sperm/mL and incubated with 1 IU neuraminidase<sup>8</sup> for  
139 30 min at 37°C. Since chicken spermatozoa were estimated to contain a maximum of 0.44  $\mu\text{mol}$   
140 sialic acid per  $10^9$  cells (Froman and Engel, 1989), and 1 IU of neuraminidase liberates 1  $\mu\text{mol}$   
141 sialic acid per minute at pH 5.0 and 37°C (unit definition for this enzyme), the conditions used  
142 would be satisfactory enough to remove terminal residues of this sugar from the sperm surface.  
143 After an initial centrifugation (700 x g, 5 min), spermatozoa were resuspended in TBS, pH 6.0,  
144 and washed once, followed by two centrifugations in TBS at a pH of 7.6. The final pellet was  
145 used immediately for lectin staining as described below (Experiments 1-3).

#### 146 Experiment 1: Flow cytometry assessment

147 Semen from each group of males was assessed twice for a total of 6 replicates. Non-  
148 treated and neuraminidase-treated spermatozoa were resuspended in TBS to a concentration of  
149  $2.5 \times 10^9$  sperm/mL. A 5  $\mu\text{L}$  aliquot of each sperm suspension was added to a 120  $\mu\text{L}$  volume of

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<sup>8</sup> Type V, from *Clostridium perfringens*; Sigma Chemical Co., St. Louis, MO

150 lectin solution to yield a final concentration of  $100 \times 10^6$  sperm/mL. A control sample was  
151 prepared by diluting non-treated spermatozoa in TBS only, and neuraminidase-treated  
152 spermatozoa also were diluted in lectin-sugar mixtures to study the specificity of binding. All  
153 samples contained  $100 \times 10^6$  sperm/mL in a final volume of 125  $\mu$ L. Samples were incubated for  
154 30 min at room temperature and protected from light. After incubation, they were centrifuged  
155 (700 x g, 5 min) 4 times and the pellets were resuspended in the appropriate buffer (e.g. TBS  
156 with or without cations, pH 7.6 or 8.0). Ten  $\mu$ L of the final resuspension of each sample were  
157 diluted in 0.5 mL of the appropriate buffer and counterstained with 12  $\mu$ M Propidium iodide<sup>9</sup>  
158 (PI) for a minimum of 5 min at room temperature.

159 A Coulter Epics XL-MCL Flow Cytometer<sup>10</sup> equipped with a single 488 nm excitation  
160 source was used for all analyses. Forward and side scatter gating were used to select single  
161 spermatozoa from clumps and debris. The fluorescence from FITC-stained and PI-stained  
162 spermatozoa was collected in FL1 (525 nm BP) and FL3 (620 nm BP) fluorescence detectors,  
163 respectively. Because cells with intact plasma membranes preclude lectins from binding to  
164 internal structures, only FITC-fluorescence signals generated by PI-negative cells were  
165 considered in the analysis. The mean FITC fluorescence intensity/cell (MnFI) of the viable sperm  
166 population was recorded from the FL1 detector output to determine lectin binding-related  
167 changes in the population.

#### 168 Experiment 2: Microscopic assessment of spermatozoa stained in suspension.

169 Spermatozoa from each group of males were assessed once for a total of 3 replicates.  
170 Non-treated and neuraminidase-treated spermatozoa were resuspended in TBS to a concentration

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<sup>9</sup> Molecular Probes, Eugene, OR

<sup>10</sup> Coulter Corporation, Miami, FL

171 of  $1 \times 10^9$  sperm/mL. A 2- $\mu$ L aliquot of each sperm suspension was added to 18  $\mu$ L of either lectin  
172 solution or lectin-sugar mixture to yield a final concentration of  $100 \times 10^6$  sperm/mL. Samples  
173 were incubated for 30 min at room temperature protected from light. After incubation, a 5  $\mu$ L  
174 aliquot was placed on a slide, fitted with a coverslip and examined using a Zeiss Axioskop  
175 microscope<sup>11</sup> equipped with an excitation filter (450-490 nm) and a barrier filter (LP520).  
176 Individual spermatozoa were evaluated for the presence/absence of fluorescence over the  
177 acrosome, nuclear region of the head, midpiece or tail at a magnification of 1000X. Only  
178 morphologically normal, whole spermatozoa were assessed. For clarity of presentation, binding  
179 that was observed in the nuclear region of the head is referred to as binding in the head region,  
180 and is not inclusive of the acrosomal region.

181 Experiment 3: Microscopic assessment of fixed spermatozoa stained on slides.

182 The experiment was conducted in triplicate with each replicate using spermatozoa from a  
183 different group of males. Non-treated and neuraminidase-treated spermatozoa were resuspended  
184 in 1 mL of 4% (wt/vol) paraformaldehyde in TBS (pH 7.2) and fixed for 30 min at room  
185 temperature. The fixed sperm suspension was then further diluted (1:20-1:30) with fixative and a  
186 10  $\mu$ L aliquot was spread onto a glass slide (1 slide/lectin) and allowed to air-dry. Following air-  
187 drying, a blocking solution (5% wt/vol bovine serum albumin in TBS) was applied to prevent  
188 non-specific background staining. Once complete evaporation of the blocking solution was  
189 observed (room temperature), selected areas on the slide were covered with 20  $\mu$ L of either the  
190 lectin solution or the lectin-sugar mixture. Slides were held at room temperature for 30 min  
191 protected from light. After incubation, slides were washed with gentle agitation (3 times, 1.5 min  
192 each) in the appropriate buffer for each lectin and allowed to air-dry protected from light. Slides

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<sup>11</sup> Carl Zeiss Inc., Thornwood, NY

193 were then mounted with ProLong Gold antifade reagent<sup>12</sup> and kept at room temperature until  
194 examination (within 2 days of staining). Individual sperm cells were evaluated as described for  
195 Experiment 2.

#### 196 Statistical analysis.

197 Values of Mean Fluorescence Intensity obtained in the flow cytometry assessment of  
198 lectin binding were compared using the Mann-Whitney U Test. Differences within a species  
199 between the control and each of the lectin-incubated samples of non-treated spermatozoa were  
200 evaluated to determine the presence ( $P < 0.05$ ) or absence ( $P > 0.05$ ) of sugar residues in the intact  
201 glycocalyx. Non-treated and neuraminidase-treated samples also were compared within a species  
202 to determine possible masking of carbohydrate residues by sialic acid. Species comparisons were  
203 made for neuraminidase-treated spermatozoa to study differences in carbohydrate content.  
204 Selective abolition of binding was evaluated by comparing the values obtained in the presence  
205 and absence of inhibitory sugars within lectins. All comparisons were made using the  
206 STATISTICA<sup>®</sup> software for Windows (release 4.5, 1993).

207

## 208 **Results**

### 209 Experiment 1: Flow cytometry assessment

210 Pre-incubation of lectins with the lowest concentrations of inhibitory sugars significantly  
211 reduced ( $P < 0.05$ ) the MnFI of all neuraminidase-treated turkey (Table 2) and chicken (Table 3)  
212 sperm samples, with the following exceptions: 1) STA and UEA-I, both species; 2) s-WGA,  
213 turkey only; and 3) PSA, GS-I, Jacalin and Lotus, chicken only. Specificity of binding  
214 subsequently was demonstrated for s-WGA in turkey spermatozoa and for GS-I and Lotus in

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<sup>12</sup> Molecular Probes, Eugene, OR

215 chicken spermatozoa with higher concentrations of inhibitory sugars (Tables 2, 3). In contrast,  
216 pre-incubation of STA, UEA-I, PSA and Jacalin with 200 or 400 mM of respective inhibitory  
217 sugars did not decrease ( $P>0.05$ ) the MnFI (data not shown), indicating that these four lectins  
218 were non-specifically binding to sperm cells. Accordingly, further experimentation was not  
219 considered for PSA and Jacalin in chicken spermatozoa, or STA and UEA-I in either species.

220 Control samples (e.g., spermatozoa incubated without lectins) emitted low levels of  
221 fluorescence (MnFI average:  $0.17\pm 0.02$ , turkey;  $0.13\pm 0.01$ , chicken). For non-neuraminidase  
222 treated turkey spermatozoa, 4 of the 15 lectins showed high ( $P<0.05$ ) MnFI (LPA,  $1.68\pm 0.53$ ;  
223 LFA,  $2.36\pm 0.60$ ; Con A,  $2.52\pm 1.05$ ; RCA-I,  $38.42\pm 9.70$ ) compared to control samples.  
224 Similarly, 5 of the 13 lectins incubated with non-neuraminidase treated chicken spermatozoa  
225 showed high ( $P<0.05$ ) MnFI (LPA,  $3.96\pm 1.66$ ; LFA,  $2.98\pm 1.21$ ; Con A,  $2.88\pm 0.67$ ; s-WGA,  
226  $4.07\pm 1.35$ ; RCA-I,  $54.58\pm 33.70$ ) compared to control samples. The MnFI for the remaining  
227 lectins incubated with non-neuraminidase treated spermatozoa was low (range: 0.20-0.35,  
228 turkey; 0.23-0.73, chicken) but still significantly different ( $P<0.05$ ) from control values with the  
229 exception of GS-II ( $0.21\pm 0.03$ ) and Lotus ( $0.20\pm 0.05$ ) in turkey samples.

230 The MnFI for non-treated and neuraminidase-treated turkey and chicken spermatozoa are  
231 shown in Tables 2 and 3. Because the enzyme neuraminidase cleaves terminal sialic acid  
232 residues, the MnFI for neuraminidase-treated spermatozoa was lower ( $P<0.05$ ) than non-treated  
233 spermatozoa incubated with LPA or LFA. Treatment of both turkey and chicken spermatozoa  
234 with neuraminidase increased ( $P<0.05$ ) the MnFI for all remaining lectins except s-WGA in  
235 chicken spermatozoa. Lectins RCA-I, WFA, and ECA yielded the highest MnFI values for both  
236 turkey and chicken neuraminidase-treated spermatozoa (Figure 1). Similar MnFI ( $P>0.05$ ) were  
237 detected for most lectins incubated with either neuraminidase-treated turkey or chicken

238 spermatozoa (Figure 1). Exceptions included sWGA and ECA, which had higher MnFI ( $P < 0.05$ )  
239 in chicken spermatozoa than turkey spermatozoa, and WFA, which had higher MnFI ( $P < 0.05$ ) in  
240 turkey spermatozoa than chicken spermatozoa (Figure 1).

241 Experiment 2: Microscopic assessment of spermatozoa stained in suspension.

242 Fluorescence data for spermatozoa stained in suspension with lectins are shown in Table  
243 4. The proportion of stained cells varied among lectins; however, virtually all spermatozoa were  
244 stained after incubation with RCA-I, WFA, LFA and LPA, as well as s-WGA in chicken  
245 spermatozoa only. Binding was typically seen as spots of fluorescence in the indicated regions.  
246 Additionally, sperm agglutination was observed in some samples as a further indication of lectin  
247 binding. With a few exceptions, lectin binding was not observed in morphologically-normal  
248 turkey or chicken spermatozoa unless the cells were pre-treated with neuraminidase. RCA-I was  
249 the most notable exception in that non-treated turkey and chicken spermatozoa were highly  
250 agglutinated and displayed bright fluorescence over the entire cell, the intensity of which  
251 increased after neuraminidase treatment. The specificity of RCA-I binding was confirmed by the  
252 complete absence of fluorescence in sperm cells incubated with RCA-I+100mM lactose. Non-  
253 and neuraminidase-treated chicken spermatozoa also exhibited fluorescence over the entire cell  
254 after incubation with s-WGA that was abolished in the presence of 2.5  $\mu\text{g/mL}$  chitin. Non-  
255 treated turkey spermatozoa incubated with Con A exhibited carbohydrate-specific binding over  
256 the acrosome and over the entire cell after neuraminidase treatment.

257 Similar staining patterns were observed for neuraminidase-treated turkey and chicken  
258 spermatozoa incubated with GNA, GS-II, WFA, ECA or Lotus, and the lectin binding was  
259 specifically inhibited by the competing sugars (Table 4). Fluorescence was detected exclusively  
260 over the acrosome region after incubation with GNA and GS-II, while binding sites for WFA and

261 ECA were distributed all over the cell. Lotus lectin binding also was observed over the entire  
262 cell; however, the degree of Lotus binding differed slightly in that chicken spermatozoa were  
263 clearly stained in the acrosome while the remaining morphological regions were covered by a  
264 cloud of diffused fluorescence, whereas binding in turkey spermatozoa was observed over the  
265 whole surface as spots of fluorescence. The staining patterns with SBA also differed between  
266 species; with chicken spermatozoa fluorescing over the entire cell, while binding was limited in  
267 the acrosome and head regions of turkey spermatozoa.

268 Sperm cellular debris (i.e. detached heads or tails) frequently was stained, even in the  
269 absence of lectin binding in morphologically normal spermatozoa. For example, with GS-I, PNA  
270 and Jacalin, normal spermatozoa were basically unstained in both non-treated and  
271 neuraminidase-treated samples; however, a large percentage of detached acrosomes and whole  
272 cells with decondensed heads were observed showing homogeneous and bright fluorescence.

### 273 Experiment 3: Microscopic assessment of fixed spermatozoa stained on slides.

274 In contrast to the binding patterns observed for spermatozoa stained in suspension, some  
275 measure of fluorescence was observed for each lectin when spermatozoa were fixed, air-dried  
276 and incubated with lectin, regardless of neuraminidase treatment. For example, GS-I bound to  
277 the acrosomal and head regions of all turkey and chicken spermatozoa observed on slides;  
278 whereas binding was minimal (observed only in abnormal cells and cellular debris) when this  
279 lectin was incubated with spermatozoa in suspension. A similar staining pattern was observed for  
280 Jacalin and PNA with the slide technique, although considerably fewer cells overall exhibited  
281 fluorescence. More importantly, Jacalin and PNA binding observed in fixed cells were not  
282 abolished by the respective inhibitory sugars, and the inhibitory sugars for GS-I only reduced  
283 fluorescence intensity at high molar concentrations (400 mM). Similarly, Con A, GNA, PSA,

284 ECA and Lotus all were observed to bind the head region of non-treated turkey and chicken  
285 spermatozoa, and this binding was not inhibited by any of the competitive sugars. Binding  
286 patterns associated with LPA and LFA were different despite the fact that both have affinity for  
287 sialic acid. LPA stained only the head region, whereas LFA binding was observed over the  
288 whole sperm surface. Surprisingly, neuraminidase-treated spermatozoa also were seen stained,  
289 and changes in the staining characteristics of these cells were not evident.

290 Binding was observed in the head region of turkey spermatozoa stained on slides with  
291 ECA irrespective of neuraminidase treatment, and this fluorescence was not abolished by the  
292 inhibitory sugar. For chicken spermatozoa, neuraminidase treatment increased the occurrence of  
293 ECA binding from only the head region to the entire cell. In this case, however, binding in the  
294 acrosome, midpiece and tail were abolished by the inhibitory sugar lactose. For turkey  
295 spermatozoa, a similar shift in the RCA-I, SBA- and WFA-binding patterns occurred between  
296 non- and neuraminidase-treated spermatozoa, and the fluorescence of all regions except the head  
297 also was inhibited in the presence of competing sugars. Regions stained by GS-II included the  
298 acrosome and head regions in non-treated turkey spermatozoa but only the head region in non-  
299 treated chicken spermatozoa. Binding extended to acrosome in the neuraminidase-treated  
300 chicken spermatozoa, with no change being observed in turkey. For both species, abolition of  
301 binding occurred only in the acrosome region with the inhibitory sugar.

302 The lectin s-WGA displayed different fluorescent intensity in non- and neuraminidase-  
303 treated spermatozoa. In general, binding was observed over acrosomal and head regions in a few  
304 cells with the majority unstained. The intensity of fluorescence was low in non-treated  
305 spermatozoa and, while neuraminidase treatment slightly increased the number of stained  
306 spermatozoa and the intensity of fluorescence, no change in binding sites was observed.

307 Fluorescence intensity was clearly reduced or not observed when spermatozoa were incubated  
308 with lectin-sugar mixtures.

309

## 310 **Discussion**

311 Here we provide the first comprehensive characterization of the carbohydrate groups  
312 comprising the glycocalyx of poultry spermatozoa. Our approach was to first delineate the types  
313 of carbohydrates present using flow cytometry and, secondly, to localize specific carbohydrate  
314 residues with respect to the morphological regions of the sperm cell. For this second objective,  
315 two methods of lectin labelling (unfixed spermatozoa in suspension versus fixed spermatozoa  
316 air-dried on slides) were compared. Because specificity of lectin binding is an important  
317 consideration, control procedures were used to verify accurate data interpretation. These  
318 procedures, including competitive inhibition with free sugars and enzymatic removal of specific  
319 carbohydrates, should significantly decrease or abolish lectin binding (Stoddart and Jones, 1998).  
320 For flow cytometry, counterstaining with an impermeable nuclear stain, such as propidium  
321 iodide, and analyzing the fluorescence signals of intact cells ensures that lectin binding is  
322 localized to the cell surface rather than intracellular sites (Ashworth et al, 1995). By adopting  
323 these types of strategies, we have identified the specific carbohydrates associated with the  
324 glycocalyx of turkey and chicken spermatozoa, although the precise distribution of these  
325 carbohydrates over the various morphological regions requires further clarification.

326 Flow cytometry assessment of lectin binding revealed that the glycocalyx of turkey and  
327 chicken spermatozoa in its physiological state (e.g. not enzymatically treated with  
328 neuraminidase) is composed mainly of glycoconjugates containing  $\beta$ -galactose,  $\alpha$ -mannose/ $\alpha$ -  
329 glucose and sialic acid as terminal saccharides, based on the high fluorescence intensity values

330 obtained with RCA-I, Con A, LFA and LPA. Some lectins that recognize terminal  $\beta$ -galactose  
331 (PNA),  $\alpha$ -mannose (GNA) and  $\alpha$ -glucose and/or  $\alpha$ -mannose (PSA) residues, however, provided  
332 low fluorescence intensity despite the fact that they recognize the same broad carbohydrate  
333 groups as RCA-I and Con A. This apparent conflict in carbohydrate identification is the result of  
334 the highly specific binding sites recognized by each lectin. It is known that lectins with a same  
335 nominal sugar binding specificity (e.g. “fucose-binding lectins”, “galactose-binding lectins”) can  
336 react quite differently with complex sugar structures (Leathem and Brooks, 1998). For example,  
337 PNA can react strongly with a disaccharide known as the “T-antigen” [Gal( $\beta$ 1,3)GalNAc].  
338 Factors affecting binding site access, such as steric configurations, are important for lectin  
339 binding (Koehler, 1978) and subtle differences in binding specificity within carbohydrate groups  
340 also exist. For example, unlike most mannose-specific lectins, GNA does not react with glucose  
341 while PSA does not recognize branched mannose structures. These examples illustrate the  
342 possibility that a diversity of glycoconjugates containing unique saccharide structures or  
343 sequences exist in the sperm surface, and a wide spectrum of lectins is necessary to properly  
344 characterize glycocalyx composition.

345         Few residues of  $\alpha$ -galactose, N-acetyl-galactosamine, N-acetyl-lactosamine and fucose,  
346 as well as terminal monomers of N-acetyl-glucosamine (also dimers in turkey spermatozoa) were  
347 detected by flow cytometry in non-neuraminidase-treated spermatozoa, initially suggesting that  
348 these carbohydrates were minor components of the glycocalyx. Enzymatic removal of terminal  
349 sialic acid residues with neuraminidase, however, demonstrated that this sugar was masking the  
350 majority of the carbohydrate residues in the sperm glycocalyx. While  $\beta$ -galactose and  $\alpha$ -  
351 mannose/ $\alpha$ -glucose residues were still abundant in neuraminidase-treated spermatozoa, residues  
352 of N-acetyl-galactosamine, N-acetyl-lactosamine and N-acetyl-glucosamine became

353 predominant carbohydrate residues for both species after terminal sialic acid residues were  
354 removed. Interestingly, the extent to which sialic acid masked the carbohydrate residues varied.  
355 Residues recognized by s-WGA in the chicken, for instance, did not appear to be significantly  
356 sialylated, and only a few of those recognized by Con A were masked. The extent of sialylation  
357 for the rest of residues was directly related to their relative abundance in the glycocalyx.

358 Sialic acids act as masking agents on antigens, receptors and other recognition sites of the  
359 cell surface (Schauer, 1985); therefore, the observation that a considerable amount of sperm  
360 surface saccharides were coated with sialic acid molecules is not surprising. This phenomenon  
361 has been observed in other species (Cooper et al, 2001), and it appears that the amount of sialic  
362 acid in sperm cells is considerably higher than that in somatic cells (Diekman, 2003). In  
363 mammalian spermatozoa, this masking of terminal sugars seems to be an integral step during  
364 sperm maturation, as sialic acid groups appear on the sperm surface as spermatozoa proceed  
365 through the epididymis (Holt, 1980). The occurrence of a similar mechanism for maturation of  
366 poultry spermatozoa needs to be verified, in part, because of the lack of a subdivided epididymal  
367 structure in the avian reproductive tract and known differences in post-testicular changes  
368 between mammalian and avian spermatozoa (Esponda and Bedford, 1985). Regardless of the  
369 mechanism for sialic acid deposition, the functional significance of the extensive sialylation of  
370 the sperm glycocalyx may be similar for both mammalian and avian species. Removal of surface  
371 sialic acid residues may increase antigenicity of chicken spermatozoa in the vagina of the hen,  
372 resulting in their destruction by an immunologically-based sperm-selection mechanism (Steele  
373 and Whishart, 1996); a similar selective mechanism has been proposed for mammalian  
374 spermatozoa (Holt, 1980). If this also applies to the turkey species, then the high extent of  
375 sialylation observed in the poultry sperm glycocalyx could be explained on the basis of that

376 immunoprotective effect of sialic acid. Moreover, the different levels of sialylation observed  
377 among sugar residues may be related to differences in the antigenicity of the glycoconjugates.

378         The most remarkable differences in glycocalyx composition between the two species  
379 were observed for N-acetyl-glucosamine dimers (s-WGA) and N-acetyl-galactosamine (WFA),  
380 with the former being more prevalent in chicken spermatozoa and the latter more prevalent in  
381 turkey spermatozoa. Another interesting species difference is that chicken spermatozoa  
382 contained higher levels of N-acetyl-lactosamine (ECA) than turkey spermatozoa.

383         Other aspects of poultry sperm glycocalyx composition can be discerned based on  
384 binding specificity. For instance, neither turkey nor chicken sperm glycocalyx appeared to  
385 contain N-acetyl-glucosamine oligomers, as specific binding for STA could not be demonstrated.  
386 Likewise, distinctive  $\alpha$ -linkages also exist within fucose residues as Lotus, rather than UEA-I,  
387 was the only “fucose-binding” lectin which specifically bound to the sperm membrane. Also  
388 interesting was that PSA ( $\alpha$ -mannose/glucose) and Jacalin ( $\alpha$ -galactose) did not bind specifically  
389 to the chicken sperm membrane; whereas low but specific binding of these lectins was evident in  
390 turkey spermatozoa.

391         The two techniques used for microscopic determination of lectin binding sites gave quite  
392 different staining patterns, most notably with non-neuraminidase treated spermatozoa. This  
393 dichotomy has been reported for other species when comparing lectin binding patterns of unfixed  
394 and fixed spermatozoa (Kallajoki et al, 1985; Navaneetham et al, 1996), as well as when  
395 comparing binding patterns after different methods of fixation (Gabriel et al, 1994). It has been  
396 suggested that procedures like fixation and air-drying disrupt the sperm plasma membrane and  
397 thereby expose acrosomal and other intracellular glycoconjugates, which results in false positive  
398 glycocalyx lectin binding (Kallajoki et al, 1985); paradoxically, a number of publications have

399 based glycoalyx characterizations on methods employing fixed and/or air-dried spermatozoa. In  
400 our study, we compared the two methods of staining poultry spermatozoa for our own  
401 verification of the suitability of using fixed, air-dried cells for microscopic localization of lectin  
402 binding. The fact that a majority of lectins appeared to bind to spermatozoa when stained on  
403 slides but not when stained in suspension is consistent with the idea that intracellular lectin  
404 binding could be occurring. Moreover, this binding was not easily inhibited and showed clear  
405 inconsistencies with the flow data (i.e. changes in staining characteristics after neuraminidase  
406 treatment were not evident for most lectins; s-WGA hardly stained a few cells in non-treated  
407 chicken spermatozoa), as well as inconsistencies in some binding patterns. For example, no sialic  
408 acid residues appeared to exist over the midpiece or tail according to LPA binding pattern with  
409 the slide method; however, it is clear that the carbohydrate actually was present in those  
410 morphological regions as neuraminidase treatment exposed masked carbohydrates detected by  
411 ECA, SBA, WFA and RCA-I. Therefore, lectin staining of fixed, air-dried cells most likely does  
412 not represent the true surface binding pattern for poultry spermatozoa.

413         Data from neuraminidase-treated spermatozoa stained in suspension revealed that the  
414 entire cell surface was coated with sialic acid and that certain glycoconjugates were segregated  
415 into membrane domains. In particular, glycoconjugates containing terminal monomers of N-  
416 acetyl-glucosamine, as well as  $\alpha$ -mannose residues recognized by GNA, appeared to be  
417 distributed only over the acrosomal region in both species. For turkey spermatozoa, N-acetyl-  
418 galactosamine residues recognized by SBA were localized over the entire head region, including  
419 the acrosome. Segregation into domains was less evident for other carbohydrates, which seemed  
420 to be distributed over the whole surface. The distribution of Con A- and Lotus-recognized  
421 residues over certain areas of the chicken spermatozoa was not clear and requires further study

422 using techniques of superior resolution. In general, lectins which demonstrated binding in  
423 unfixed spermatozoa also were delineated by flow cytometry. However, binding sites for the  
424 lectins s-WGA, Jacalin and PSA, which exhibited low fluorescence intensities in conjunction  
425 with flow cytometry analysis of turkey spermatozoa, were not evident during microscopic  
426 evaluation. Further studies are then also necessary to clarify the absence of binding sites  
427 observed for these lectins in turkey spermatozoa, as well as for GS-I and PNA in both species.

428         From a comparative standpoint, the use of lectins to identify glycoconjugates in unfixed,  
429 mature/ejaculated spermatozoa from other species highlights the diversity found in the sperm  
430 glycocalyx. For example, while sialic acid and galactose residues clearly were abundant in the  
431 glycocalyx of poultry spermatozoa, these major carbohydrate groups are not surface components  
432 of *Drosophila* spermatozoa (Perotti and Pasini, 1995). Conversely, fucose residues did not  
433 appear to be a major component of the poultry sperm glycocalyx, whereas the glycocalyx of  
434 human spermatozoa has been reported to contain 30% fucose (Calzada et al, 1994). Similar to  
435 poultry, the glycocalyx of rabbit spermatozoa does not contain UEA-I-recognized fucose  
436 residues, but has been reported to contain sialic acid, N-acetyl-glucosamine, N-acetyl-  
437 galactosamine, galactose, glucose and mannose (Nicolson and Yanagimachi, 1972). Also similar  
438 to chicken spermatozoa, boar and ram spermatozoa exhibited bright fluorescence when incubated  
439 with lectins RCA-I and s-WGA; however, unlike poultry spermatozoa, the glycocalyx of porcine  
440 and ovine spermatozoa demonstrated high fluorescence after incubation with the lectins STA  
441 and Jacalin (Ashworth et al, 1995). Ram spermatozoa also differ from poultry spermatozoa by  
442 exhibiting higher fluorescence intensity after incubation with the lectin PNA (Magargee et al,  
443 1988). In addition to the species-specific variability in the number of carbohydrate residues (as  
444 indicated by the level of fluorescence intensity) the distribution of carbohydrates over the sperm

445 surface also varies among species (Ashworth et al, 1995; Magargee et al, 1988; Perotti and  
446 Pasini, 1995), including chicken and turkey spermatozoa. For example, SBA recognized N-  
447 acetyl-galactosamine residues along the entire length of neuraminidase-treated chicken  
448 spermatozoa, but these same terminal carbohydrates were found only on the acrosome and head  
449 regions of turkey spermatozoa. Taken together, these data indicate that the carbohydrate content  
450 of sperm glycocalyx is highly species-specific, and suggests that delineation of the physiological  
451 basis for this diversity among species and taxa may provide new insight about sperm function.

452 In summary, the glycocalyx of turkey and chicken spermatozoa is extensively sialylated  
453 and contains residues of  $\alpha$ -mannose/ $\alpha$ -glucose,  $\alpha$ - and  $\beta$ -galactose,  $\alpha$ -fucose,  $\alpha$ - and  $\beta$ -N-acetyl-  
454 galactosamine and N-acetyl-lactosamine, as well as monomers and dimers of N-acetyl-  
455 glucosamine in variable amounts. Sugar residues specific for STA and UEA-I do not appear to  
456 exist in either of the two species; whereas those for PSA and Jacalin appear to only exist in  
457 turkey spermatozoa. Chicken spermatozoa are considerably richer in dimers of N-acetyl-  
458 glucosamine and residues of N-acetyl-lactosamine than turkey spermatozoa; whereas turkey  
459 spermatozoa contain a higher amount of WFA-recognized N-acetyl-galactosamine residues.  
460 Monomers of N-acetyl-glucosamine and glycoconjugates containing GNA-recognized mannose  
461 residues appear to be clearly restricted to the acrosomal region in both turkey and chicken  
462 spermatozoa.

463 In the light of the findings reported here, it becomes evident that the glycocalyx of  
464 poultry spermatozoa is generally similar to that of mammalian spermatozoa in that (1) it contains  
465 the same types of carbohydrates reported for mammals, (2) there is a phenomenon of sialylation  
466 in terminal sugars with apparently the same functional purpose, (3) the membrane is structurally  
467 subdivided into regional domains, and (4) species-specificity exists with regard to composition

468 and distribution of carbohydrates. Known physiological differences existing between  
469 mammalian and avian spermatozoa, such as the fertilizing ability of testicular avian spermatozoa  
470 (Howarth, 1983) without a requirement for capacitation (Howarth, 1970), suggest that even  
471 similar glycoconjugates may have widely differing functional relevance. In mammalian  
472 spermatozoa, for example, glycoconjugates recognized by the WGA lectin have been shown to  
473 participate actively in the phenomenon of capacitation (Mahmoud and Parrish, 1996); however,  
474 the role of WGA-recognized glycoconjugates in poultry spermatozoa would not be expected to  
475 be associated with this physiological event. Likewise, N-acetyl-galactosamine residues appear to  
476 play a role in the development of hyperactive motility in mammalian spermatozoa (Bergersson et  
477 al, 1994; Kawakami et al, 2002); whereas hyperactivation is not observed in poultry  
478 spermatozoa. Characterizing the carbohydrate residues in the turkey and chicken sperm  
479 glycocalyx represents the first step in our investigation. Studies currently are underway to  
480 determine the functional significance of these carbohydrates with respect to conventional semen  
481 storage methodologies.

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488

488           **References**

489           Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD, eds. *Molecular Biology of*  
490 *the Cell*. 3<sup>rd</sup> ed. New York: Garland Publishing; 1994.

491           Ashworth PJC, Harrison RAP, Miller NGA, Plummer JM, Watson PF. Flow cytometric  
492 detection of bicarbonate-induced changes in lectin binding in boar and ram sperm populations.  
493 *Mol Reprod Dev* 1995;40:164-176.

494           Bakst MR, Howarth B. The effect of glycerol and its removal on cock spermatozoa  
495 concanavalin A and cationized ferritin binding sites. *Poult Sci* 1977;56:1318-1323.

496           Bearer FL, Friend DS. Morphology of mammalian sperm membranes during  
497 differentiation, maturation, and capacitation. *J Electron Microsc Tech* 1990;16:281-297.

498           Bergerson W, Amselgruber W, Sinowatz F, Bergerson M. Morphological evidence of  
499 sperm maturation in the ampulla ductus deferentis of the bull. *Cell Tissue Res* 1994;275:537-541.

500           Burrows WH, Quinn JP. The collection of spermatozoa from the domestic fowl and  
501 turkey. *Poult Sci* 1937;14:251-254.

502           Calzada L, Salazar EL, Pedron N. Presence and chemical composition of glycoproteic  
503 layer on human spermatozoa. *Arch Androl* 1994;33:87-92.

504           Cooper NJ, McClean RV, Leigh CM, Breed WG. Glycoconjugates on the surface of  
505 epididymal spermatozoa in a marsupial, the brushtail possum, *Trichosurus vulpecula*.  
506 *Reproduction* 2001;122:165-176.

507           Diekman AB. Glycoconjugates in sperm function and gamete interactions: how much  
508 sugar does it take to sweet-talk the egg? *Cell Mol Life Sci* 2003;60:298-308.

509 Eddy EM. The spermatozoon. In: Knobil E, Neill JD, Ewing LL, Markert CL, Greenwald  
510 GS, Ptuff DW, eds. *The Physiology of Reproduction*. Vol 1. New York: Raven Press; 1988: 27-  
511 68.

512 Esponda P, Bedford JM. Surface of the rooster spermatozoon changes in passing through  
513 the wolffian duct. *J Exp Zool* 1985;234:441-449.

514 Froman DP, Engel HN. Alteration of the spermatozoal glycocalyx and its effect on  
515 duration of fertility in the fowl (*Gallus domesticus*). *Biol Reprod* 1989;40:615-621.

516 Froman DP, Feltmann AJ. Sperm mobility: a quantitative trait of the domestic fowl  
517 (*Gallus domesticus*). *Biol Reprod* 1998;58:379-384.

518 Froman DP, McLean AJ. Objective measurement of sperm motility based upon sperm  
519 penetration of Accudenz. *Poult Sci* 1996;75:776-784.

520 Froman DP, Thurston RJ. Decreased fertility resulting from treatment of fowl  
521 spermatozoa with neuraminidase or phospholipase c. *Poult Sci* 1984;63:2479-2482.

522 Gabriel LK, Franken DR, Van Der Horst G, Kruger TF. Localization of wheat germ  
523 agglutinin lectin receptors on human sperm by fluorescence microscopy: utilization of different  
524 fixatives. *Arch Androl* 1994;33:77-85.

525 Holt WV. Surface-bound sialic acid on ram and bull spermatozoa: deposition during  
526 epididymal transit and stability during washing. *Biol Reprod* 1980;23:847-857.

527 Howarth B. An examination for sperm capacitation in the fowl. *Biol Reprod* 1970;3:338-  
528 341

529 Howarth B. Fertilizing ability of cock spermatozoa from the testis, epididymis and vas  
530 deferens following intramaginal insemination. *Biol Reprod* 1983;28:586-590

531 Kallajoki M, Malmi R, Virtanen I, Suominen J. Glycoconjugates of human sperm  
532 surface. A study with fluorescent lectin conjugates and *Lens culinaris* agglutinin affinity  
533 chromatography. *Cell Biol Int Rep* 1985;9:151-164.

534 Kawakami E, Morita Y, Hori T, Tsutsui T. Lectin-binding characteristics and  
535 capacitation of canine epididymal spermatozoa. *J Vet Med Sci* 2002;64:543-549.

536 Koehler JK. The mammalian sperm surface: studies with specific labeling techniques. *Int*  
537 *Rev Cytol* 1978;54:73-108.

538 Koehler JK. Lectins as probes of the spermatozoon surface. *Arch Androl* 1981;6:197-217.

539 Leatham AJ, Brooks SA. Light microscopy. In: Rhodes JM, Milton JD, eds. *Methods in*  
540 *Molecular Medicine, Vol. 9: Lectin Methods and Protocols*. Totowa: Humana Press Inc; 1998:3-  
541 20.

542 Long JA, Kramer M. Effect of vitamin E on lipid peroxidation and fertility after artificial  
543 insemination with liquid-stored turkey semen. *Poult Sci* 2003;82:1802-1807.

544 Magargee SF, Kunze E, Hammerstedt RH. Changes in lectin-binding features of ram  
545 sperm surfaces associated with epididymal maturation and ejaculation. *Biol Reprod*  
546 1988;38:667-685.

547 Mahmoud AI, Parrish JJ. Oviduct fluid and heparin induce similar surface changes in  
548 bovine sperm during capacitation: a flow cytometric study using lectins. *Mol Reprod Dev*  
549 1996;43:554-560.

550 McLean DJ, Feltmann AJ, Froman DP. Transfer of sperm into a chemically defined  
551 environment by centrifugation through 12% (wt/vol) Accudenz. *Poult Sci* 1998;77:163-168.

552 Navaneetham D, Sivashanmugam P, Rajalakshmi M. Changes in binding of lectins to  
553 epididymal, ejaculated and capacitated spermatozoa of the Rhesus monkey. *Anat Rec*  
554 1996;245:500-508.

555 Nicolson GL, Yanagimachi R. Terminal saccharides on sperm plasma membranes:  
556 identification by specific agglutinins. *Science* 1972;177:276-279.

557 Perotti ME, Pasini ME. Glycoconjugates of the surface of the spermatozoa of *Drosophila*  
558 *melanogaster*: a qualitative and quantitative study. *J Exp Zool* 1995;271:311-318.

559 Rademacher TW, Parekh RB, Dwek RA. Glycobiology. *Annu Rev Biochem* 1988;57:785-  
560 838.

561 Rojas MV, Esponda P. Plasma membrane glycoproteins during spermatogenesis and in  
562 spermatozoa of some fishes. *J Submicrosc Cytol Pathol* 2001;33:133-140.

563 Schauer R. Sialic acids and their role as biological masks. *Trends Biochem Sci*  
564 1985;10:357-360.

565 Schröter S, Osterhoff C, McArdle W, Ivell R. The glycocalyx of the sperm surface. *Hum*  
566 *Reprod Update* 1999;5:302-313.

567 Steele MG, Wishart GJ. Demonstration that the removal of sialic acid from the surface of  
568 chicken spermatozoa impedes their transvaginal migration. *Theriogenology* 1996;46:1037-1044.

569 Stoddart RW, Jones CJP. Lectin histochemistry and cytochemistry: light microscopy. In:  
570 Rhodes JM, Milton JD, eds. *Methods in Molecular Medicine, Vol. 9: Lectin Methods and*  
571 *Protocols*. Totowa: Humana Press Inc; 1998:21-39.

572 Varki A. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology*  
573 1993;3:97-130.

574 Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD, Ewing LL, Markert CL,  
575 Greenwald GS, Pfaff DW, eds. *The Physiology of Reproduction*. Vol 1. New York: Raven Press;  
576 1988:135-185.  
577

577 **Table 1.** Carbohydrate affinities and inhibitory sugars for the 17 FITC-lectins.

Lectin	Carbohydrate <sup>1</sup> affinity	Inhibitory sugar <sup>2</sup>
GS-I	$\alpha$ -Gal	Galactose
Jacalin	$\alpha$ -Gal	Galactose
RCA-I	$\beta$ -Gal	Lactose
PNA	$\beta$ -Gal	Lactose
Con A	$\alpha$ -Man, $\alpha$ -Glc, Branched mannose	Methyl $\alpha$ -D-mannopyranoside
PSA	$\alpha$ -Man > $\alpha$ -Glc	Methyl $\alpha$ -D-mannopyranoside
GNA	$\alpha$ -Man	Mannose
GS-II	Terminal $\alpha$ - or $\beta$ -GlcNAc	N-acetyl-glucosamine
s-WGA	GlcNAc( $\beta$ 1,4)GlcNAc > $\beta$ -GlcNAc	Chitin
STA	GlcNAc( $\beta$ 1,4)GlcNAc oligomers	Chitin
SBA	$\alpha$ - or $\beta$ -GalNAc > $\alpha$ - or $\beta$ -Gal	N-acetyl-galactosamine
WFA	$\alpha$ - or $\beta$ -GalNAc	N-acetyl-galactosamine
Lotus	$\alpha$ -Fuc	Fucose
UEA-I	$\alpha$ -Fuc	Fucose
ECA	Gal( $\beta$ 1,4)GlcNAc	Lactose
LFA	Sialic acid	not applicable
LPA	Sialic acid > GlcNAc	not applicable

578 <sup>1</sup>Abbreviations: D-galactose (Gal); D-mannose (Man); D-glucose (Glc); N-acetyl-D-glucosamine  
579 (GlcNAc); N-acetyl-D-galactosamine (GalNAc); L-fucose (Fuc); N-acetyl-lactosamine  
580 [Gal( $\beta$ 1,4)GlcNAc; LacNAc].

581 <sup>2</sup>Specific sugars used: Lactose = Gal( $\beta$ 1,4)Glc; Chitin = Poly(1 $\rightarrow$ 4)- $\beta$ -GlcNAc.

582 **Table 2.** Mean fluorescence intensity ( $\pm$ SD; n=6) of turkey spermatozoa incubated with lectins  
 583 (L) and of neuraminidase-treated turkey spermatozoa incubated with lectins (NT/L) or lectin-  
 584 sugar mixtures (NT/L+S).

Carbohydrate group	Lectins	L	NT/L	NT/L+S
Gal	GS-I	0.26 $\pm$ 0.05 <sup>1</sup>	1.25 $\pm$ 0.50 <sup>2,a</sup>	0.67 $\pm$ 0.23 <sup>b</sup>
	Jacalin	0.23 $\pm$ 0.05 <sup>1</sup>	0.72 $\pm$ 0.25 <sup>2,a</sup>	0.32 $\pm$ 0.11 <sup>b</sup>
	RCA-I	38.42 $\pm$ 9.70 <sup>1</sup>	333.95 $\pm$ 73.92 <sup>2,a</sup>	4.83 $\pm$ 2.03 <sup>b</sup>
	PNA	0.33 $\pm$ 0.07 <sup>1</sup>	1.65 $\pm$ 0.80 <sup>2,a</sup>	0.29 $\pm$ 0.07 <sup>b</sup>
Glc and/or Man	Con A	2.52 $\pm$ 1.05 <sup>1</sup>	5.11 $\pm$ 0.84 <sup>2,a</sup>	0.88 $\pm$ 0.19 <sup>b</sup>
	PSA	0.33 $\pm$ 0.04 <sup>1</sup>	0.65 $\pm$ 0.20 <sup>2,a</sup>	0.38 $\pm$ 0.17 <sup>b</sup>
	GNA	0.27 $\pm$ 0.06 <sup>1</sup>	2.03 $\pm$ 0.80 <sup>2,a</sup>	0.97 $\pm$ 0.29 <sup>b</sup>
GlcNAc	GS-II	0.21 $\pm$ 0.03 <sup>1</sup>	9.96 $\pm$ 4.77 <sup>2,a</sup>	0.51 $\pm$ 0.17 <sup>b</sup>
	s-WGA	0.35 $\pm$ 0.08 <sup>1</sup>	0.73 $\pm$ 0.28 <sup>2,a</sup>	0.33 $\pm$ 0.06 <sup>a†</sup>
GalNAc	SBA	0.28 $\pm$ 0.06 <sup>1</sup>	7.18 $\pm$ 2.70 <sup>2,a</sup>	0.38 $\pm$ 0.07 <sup>b</sup>
	WFA	0.31 $\pm$ 0.12 <sup>1</sup>	109.10 $\pm$ 35.54 <sup>2,a</sup>	0.65 $\pm$ 0.31 <sup>b</sup>
Fuc	Lotus	0.20 $\pm$ 0.05 <sup>1</sup>	1.18 $\pm$ 0.65 <sup>2,a</sup>	0.29 $\pm$ 0.04 <sup>b</sup>
LacNAc	ECA	0.29 $\pm$ 0.07 <sup>1</sup>	28.92 $\pm$ 7.86 <sup>2,a</sup>	0.50 $\pm$ 0.19 <sup>b</sup>
Sialic acid	LFA	2.36 $\pm$ 0.60 <sup>1</sup>	0.51 $\pm$ 0.04 <sup>2</sup>	not applicable
	LPA	1.68 $\pm$ 0.53 <sup>1</sup>	0.87 $\pm$ 0.50 <sup>2</sup>	not applicable

585 <sup>1,2</sup> Within the same row indicates differences (P<0.05) between L and NT/L treatments.

586 <sup>a,b</sup> Within the same row indicates differences (P<0.05) between NT/L and NT/L+S treatments.

587 <sup>†</sup> Binding inhibited (P<0.05) with 5  $\mu$ g/mL (NT/L, 0.82 $\pm$ 0.26; NT/L+S, 0.27 $\pm$ 0.05; n=3).

588

589

589 **Table 3.** Mean fluorescence intensity ( $\pm$ SD; n=6) of chicken spermatozoa incubated with lectins  
 590 (L), and of neuraminidase-treated chicken spermatozoa incubated with lectins (NT/L) or lectin-  
 591 sugar mixtures (NT/L+S).

Carbohydrate group	Lectins	L	NT/L	NT/L+S
Gal	GS-I	0.39 $\pm$ 0.23 <sup>1</sup>	1.86 $\pm$ 0.66 <sup>2,a</sup>	1.04 $\pm$ 0.35 <sup>a†</sup>
	RCA-I	54.58 $\pm$ 33.70 <sup>1</sup>	450.36 $\pm$ 55.89 <sup>2,a</sup>	5.19 $\pm$ 3.58 <sup>b</sup>
	PNA	0.46 $\pm$ 0.22 <sup>1</sup>	1.12 $\pm$ 0.36 <sup>2,a</sup>	0.22 $\pm$ 0.04 <sup>b</sup>
Glc and/or Man	Con A	2.88 $\pm$ 0.67 <sup>1</sup>	5.01 $\pm$ 1.75 <sup>2,a</sup>	0.62 $\pm$ 0.13 <sup>b</sup>
	GNA	0.65 $\pm$ 0.32 <sup>1</sup>	1.82 $\pm$ 0.30 <sup>2,a</sup>	1.22 $\pm$ 0.25 <sup>b</sup>
GlcNAc	GS-II	0.33 $\pm$ 0.19 <sup>1</sup>	13.33 $\pm$ 3.70 <sup>2,a</sup>	0.87 $\pm$ 0.46 <sup>b</sup>
	s-WGA	4.07 $\pm$ 1.35 <sup>1</sup>	4.96 $\pm$ 2.90 <sup>1,a</sup>	0.31 $\pm$ 0.18 <sup>b</sup>
GalNAc	SBA	0.64 $\pm$ 0.38 <sup>1</sup>	8.37 $\pm$ 2.17 <sup>2a</sup>	0.45 $\pm$ 0.09 <sup>b</sup>
	WFA	0.73 $\pm$ 0.36 <sup>1</sup>	40.53 $\pm$ 13.75 <sup>2a</sup>	0.63 $\pm$ 0.30 <sup>b</sup>
Fuc	Lotus	0.23 $\pm$ 0.05 <sup>1</sup>	0.52 $\pm$ 0.28 <sup>2a</sup>	0.29 $\pm$ 0.14 <sup>a*</sup>
LacNAc	ECA	0.37 $\pm$ 0.06 <sup>1</sup>	45.18 $\pm$ 16.36 <sup>2a</sup>	1.62 $\pm$ 0.75 <sup>b</sup>
Sialic acid	LFA	2.98 $\pm$ 1.21 <sup>1</sup>	0.59 $\pm$ 0.34 <sup>2</sup>	not applicable
	LPA	3.96 $\pm$ 1.66 <sup>1</sup>	1.13 $\pm$ 0.40 <sup>2</sup>	not applicable

592 <sup>1,2</sup> Within the same row indicates differences (P<0.05) between L and NT/L treatments.

593 <sup>a,b</sup> Within the same row indicates differences (P<0.05) between NT/L and NT/L+S treatments.

594 <sup>†</sup> Binding inhibited (P<0.05) at 200 mM (NT/L, 1.73 $\pm$ 0.24; NT/L+S, 1.09 $\pm$ 0.20; n=3).

595 \* Binding inhibited (P<0.05) at 200 mM (NT/L, 1.57 $\pm$ 0.65; NT/L+S, 0.23 $\pm$ 0.01; n=3).

596

596 **Table 4.** Binding patterns<sup>†</sup> observed (n=3) in lectin suspensions of non-treated spermatozoa (L),  
 597 neuraminidase-treated spermatozoa (NT/L) and neuraminidase-treated spermatozoa incubated  
 598 in the presence (NT/L +S) of inhibitory sugar.

	T U R K E Y			C H I C K E N		
	L	NT/L	NT/L+S	L	NT/L	NT/L+S
<b>GS-I<sup>1</sup></b>	nf	Nf	Nf	nf	nf	Nf
<b>Jacalin<sup>1</sup></b>	nf	Nf	Nf	---	---	---
<b>RCA-I<sup>1</sup></b>	A/H/MP/T	A/H/MP/T(↑)	X	A/H/MP/T	A/H/MP/T(↑)	X
<b>PNA<sup>1</sup></b>	nf	Nf	Nf	nf	Nf	Nf
<b>Con A<sup>2</sup></b>	A	A/H/MP/T	X	nf	A/diffuse	X
<b>PSA<sup>2</sup></b>	nf	Nf	Nf	---	---	---
<b>GNA<sup>2</sup></b>	nf	A	X	nf	A	X
<b>GS-II<sup>3</sup></b>	nf	A	X	nf	A	X
<b>s-WGA<sup>3</sup></b>	nf	Nf	Nf	A/H/MP/T	A/H/MP/T	X
<b>SBA<sup>4</sup></b>	nf	A/H	X	nf	A/H/MP/T	X
<b>WFA<sup>4</sup></b>	nf	A/H/MP/T	X	nf	A/H/MP/T	X
<b>Lotus<sup>5</sup></b>	nf	A/H/MP/T	X	nf	A/diffuse	X
<b>ECA<sup>6</sup></b>	nf	A/H/MP/T	X	nf	A/H/MP/T	X
<b>LFA<sup>7</sup></b>	A/H/MP/T	A/H/MP/T(↓)	---	A/H/MP/T	A/H/MP/T(↓)	---
<b>LPA<sup>7</sup></b>	A/H/MP	A/H/MP	---	A/H/MP	A/H/MP	---

599 <sup>†</sup> Abbreviations: acrosome (A); nuclear region of head (H); midpiece (MP); tail (T); fluorescence  
 600 intensity increased (↑)/decreased (↓); no fluorescence (nf); binding inhibited (X); not tested (---).

601 <sup>1-7</sup> Carbohydrate group: Gal<sup>1</sup>; Glc and/or Man<sup>2</sup>; GlcNAc<sup>3</sup>; GalNAc<sup>4</sup>; Fuc<sup>5</sup>; LacNAc<sup>6</sup>; Sialic acid<sup>7</sup>.

602

602 **Figure 1.** Comparison of the Mean Fluorescence Intensity (mean±SD; n=6) for neuraminidase-  
603 treated turkey and chicken spermatozoa incubated with non-sialic-acid-binding lectins. Lectins  
604 for which specific binding could not be demonstrated (chicken, PSA, Jacalin; both species, UEA,  
605 STA) are not shown. Within lectins, <sup>a,b</sup> denotes significant differences (P<0.05) between species.

