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Glycohistochemical investigation of canine and feline zonae pellucidae of preantral and antral oocytes

Francesco Parillo[1] and Andrea Verini-Supplizi[2]

[1] Istituti di Anatomia degli Animali Domestici and [2] Produzioni Animali, Facoltà di Medicina Veterinaria, Università degli Studi di Perugia, via San Costanzo 4, 06126 Perugia, Italy

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Summary

Glycoconjugate modifications were analysed in the zona pellucida during development of oocytes in dog and cat using conventional histochemical staining methods with or without previous carbohydrate digestion. A series of lectins combined with desulphation and sialic acid degradation were applied. No differences were observed between dog and cat follicles using conventional histochemical staining methods. In both species, the zona pellucida and follicular fluid/intercellular matrix strongly reacted with PAS and high iron diamine stain (HID) and reacted moderately with low iron diamine stain (LID). Treatment with testicular hyaluronidase, chondroitinase ABC, chondroitinase AC and chondroitinase B treatment diminished HID and LID positivity of follicular fluid and intercellular matrix. Lectins that gave the most intense staining of the zona pellucida of both species were SBA, PNA, RCA-I, GSA-IB4 and WGA, indicating the presence of β-D-GalNAc, D-Gal and GlcNAc residues. Sulpho- and asulpho-carbohydrates were identified in terminal and/or subterminal positions linked to sialic acid residues. In conclusion, the results indicate that glycosaminoglycans are not present in the zona pellucida of both species. Differences were observed in carbohydrate residues and in their spatial distribution, depending on species and developmental stage of the follicles. The similarity in lectin affinity between ooplasm and zona pellucida of oocytes present in follicles at different stages of development confirm the involvement of oocytes in zona pellucida production.

Correspondence to: F. Parillo

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Introduction

Histochemical investigations of the mammalian ovary have paid considerable attention to the glycoconjugate composition of the zona pellucida (ZP), a glycoprotein-containing envelope which is formed during oocyte growth (Avilés et al., 1994; Skutelsky et al., 1994; Parillo et al., 1996, 1998; Verini Supplizi et al., 1996). These studies, using lectins as carbohydrate-specific histochemical markers, revealed variability with respect to the presence and distribution patterns of oligosaccharide chains of ZP glycoconjugates, in various animal species. It was also reported that gamete recognition in most species is mediated by a complementary sperm receptor and a terminal carbohydrate of the ZP glycoproteins (Miller and Ax, 1990; Fayrer-Hosken, 1994; Wassarmann, 1994). Therefore, differences in lectin-binding may explain the species-specificity of gamete interaction in mammals.

Changes in the glycoconjugate composition of the ZP and follicular fluid during oocyte and follicle growth have been previously identified in rodents (Tadano and Yamada, 1978; Tesoriero, 1984; Delgado and Zoller, 1987) and dog (Tesoriero, 1984). These studies were carried out with conventional histochemical procedures using dyes such as PAS, cationic dyes and iron diamines which all yield incomplete information about the structure of the carbohydrate moieties of glycoconjugates (Spicer and Schulte, 1992). This prompted us to examine in dog and cat the modifications in glycoconjugate content that occur in the ZP during oocyte maturation. Conventional histochemical staining methods were applied either or not after glycolytic digestion as well as glycohistochemical methods combined with desulphation procedures and neuraminidase treatment. Because sulphated groups in ester linkages may inhibit labelling with some lectins (Lotan et al., 1975; Martinez-Menarguez et al., 1992), we performed desulphation and neuraminidase digestion in conjunction with lectin staining in order to identify sulphocarbohydrates in the terminal and/or subterminal position of the oligosaccharide side chains.

Material and Methods

Tissue collection. Canine (*Canis familiaris*, n = 5) and feline (*Felis catus*, n = 5) ovaries were obtained from sexually mature females undergoing ovariectomy. Specimens were immediately fixed in Carnoy's fluid for 24 h, postfixed in a solution of 2% calcium acetate and 4% paraformaldehyde for 3 h at room temp (Menghi, 1984; Spicer and Shulte, 1988) and then dehydrated, cleared in xylene, and embedded in paraffin wax. Serial sections (5 μm thick) were prepared and stained with conventional histochemical methods and lectin histochemical methods as described below.

Conventional histochemical staining methods. Sections were stained with periodic-acid Schiff (PAS), high iron diamine (HID) and low iron diamine (LID) (2 sections/treatment/ovary). Before LID and HID staining, adjacent serial sections were incubated with enzymes that specifically remove glycosaminoglycans. The enzymes used were: a) testicular hyaluronidase (Leppi and Stoward, 1965); b) *Streptomyces* hyaluronidase (Yamada and Hirano, 1973); c) chondroitinase ABC (Yamada, 1974); d) chondroitinase AC (1 U/ml in 0.1 M Tris-HCl buffer pH 8.0 for 4 h at 37 °C); e) chondroitinase B (10 U/ml in 0.1 M Tris-HCl buffer pH 8.0 for 4 h at 37 °C); f) heparitinase (heparinase III; 5 U/ml in 0.1 M sodium acetate pH 7.0 for 4 h at 40 °C).

Lectin histochemistry. Sections were processed for lectin histochemistry according to the procedures that were previously described by Shulte and Spicer (1983) with slight modifications. After blocking endogenous peroxidase by incubation with a solution containing 0.3% H_2O_2-methanol for 30 min at room temp, sections were rinsed in phosphate-buffered saline (PBS) and incubated (2 sections/lectin/ovary) in a lectin-horseradish peroxidase (HRP) conjugate solution (in the same buffer) for 1 h at room temp in a humidified chamber. The HRP-lectin conjugates, their hapten sugars and the appropriate concentrations which gave maximum staining intensity with minimum background are listed in Table 1. Sections were then rinsed in PBS and lectin binding sites were visualised by incubation in a solution containing 3,3′-diaminobenzidine-H_2O_2 for 10 min at room temp. After rinsing in distilled water, sections were dehydrated and mounted in Canada balsam (BDH, Milano, Italy). Some lectin staining procedures were preceded by neuraminidase (type V from *Clostridium perfringens*) digestion, that was carried out during 36 h at 37 °C in a solution of 0.1 M acetate buffer, pH 5.5, and 10 mM $CaCl_2$ containing the enzyme at a concentration of 0.86 U/ml. The O-acyl substituents at C4 of sialic acids, which cause resistance to *C. perfringens* neuraminidase, were removed by immersing paraffin sections in 1% potassium hydroxide solution in 70% ethanol for 15 min prior to enzymatic degradation.

Table 1. Lectins that were applied in the present study and corresponding carbohydrate specificity

Source of lectin	Acronym	Inhibitory sugars[1]	Lectin concentration (μg/ml)
Arachis hypogaea	PNA	β-D-Gal-(1 → 3)-D-GalNAc	40
Griffonia simplicifolia	GSA-II	α and β GlcNAc	50
Griffonia simplicifolia IB4	GSA-IB4	α-D-Gal	20
Ulex europaeus	UEA-I	α-L-Fuc	20
Lotus tetragonolobus	LTA	α-L-Fuc	20
Dolichos biflorus	DBA	α-D-GalNAc	10
Glycine max	SBA	α-D-GalNAc > β-D-GalNAc	10
Triticum vulgare	WGA	GlcNAc > sialic acid	10
Canavalia ensiformis	Con-A	α-D-Man < α-D-Glc	20
Lens culinaris	LCA	α-D-Man > α-D-Glc	50
Ricinus communis	RCA-I	β-D-Gal-(1 → 4)-D-GlcNAc	50

[1] β-D-Gal = β-D-galactose; α-D-Gal = α-D-galactose; D-GalNAc = D-N-acetylgalactosamine; α-D-GalNAc = α-D-N-acetylgalactosamine; β-D-GalNAc = β-D-N-acetylgalactosamine; GlcNAc ≐ N-acetylglucosamine; α-L-Fuc = α-L-fucose; α-D-Man = α-D-mannose; α-D-Glc = α-D-glucose.

All HRP-labelled lectins and enzymes were purchased from Sigma (St. Louis, MO, USA) with the exception of LCA-lectin which was obtained from ICN Biomedicals (Costa Mesa, CA, USA).

Before lectin staining and neuraminidase digestion, some sections were subjected to a desulphation procedure consisting of sequential methylation-saponification which was performed by dipping the slides in 0.15 N HCl in methanol (5 h, 60 °C) and then in a 1% solution of potassium hydroxide (KOH) in 70% ethanol for 15 min at room temp (Martinez-Menarguez et al., 1992).

Controls. Controls for lectin histochemistry were carried out by either incubating the samples in a solution containing lectins and the appropriate competing sugar (0.2–0.4 M) or by omitting the lectin HRP-conjugates. Controls for enzymatic digestion were performed by treating the sections with the respective buffer solutions without enzyme. The effectiveness of desulphation technique was demonstrated by staining with HID.

Results

Canine and feline follicles were classified according to Pedersen and Peters (1968). In the present study, reactivity of ZP, ooplasm, granulosa cells and follicular fluid/intercellular matrix with respect to complex carbohydrates was evaluated in preantral (stages 3 b, 4, 5 a, 5 b) and antral follicles (stages 6, 7; $\varnothing \leq 0.5$ mm). The most relevant results obtained with conventional and lectin histochemistry are listed in Tables 2, 3 and 4.

Conventional histochemistry. No differences were observed between dog

Table 2. Intensity of PAS, HID and LID staining in canine and feline ovarian follicles[1]

Treatment	Zona pellucida	Follicular fluid and intercellular matrix	Ooplasm and granulosa cells
PAS	+++	+++	–
HID	+++	+++	–
Testicular hyaluronidase/HID	+++	+	–
Bacterial hyaluronidase/HID	+++	+++	–
Chondroitinase ABC/HID	+++	+	–
Chondroitinase AC/HID	+++	+	–
Chondroitinase B/HID	+++	+	–
Heparitinase/HID	+++	++	–
LID	++	++	–
Testicular hyaluronidase/LID	++	–	–
Bacterial hyaluronidase/LID	++	++	–
Chondroitinase ABC/LID	++	+	–
Chondroitinase AC/LID	++	+	–
Chondroitinase B/LID	++	+	–
Heparitinase/LID	++	++	–

[1] (+) and (–) indicate staining intensity on a semi-quantitative scale: (–) negative, (+) weak, (++) moderate and (+++) strong reaction.

Table 3. Lectin positivity of canine preantral and antral follicles[1]

Lectin and treatment[2]	Follicular stage	Zona pellucida			Ooplasm	Granulosa cells	Follicular fluid and intercellular matrix
		Uniform[3]	Internal	External			
S/SBA	(3 b–4)	+++			–	–	
	(5 a–5 b)		+	+++	–	–	
	(6–7)		+	+++	–	–	–
D/SBA	(3 b–4)	+			–	–	
	(5 a–5 b)		+	+	–	–	
	(6–7)		+	+	–	–	++
D/S/SBA	(3 b–4)	+++			+	+	
	(5 a–5 b)		+	+++	+	+	
	(6–7)		+	+++	+	+	+++
S/PNA	(3 b–4)	–	–	–	–	–	
	(5 a–5 b)	–	–	–	–	–	
	(6–7)	–	–	–	–	–	++
D/S/PNA	(3 b–4)	+++			+	+	
	(5 a–5 b)	+++			+	+	
	(6–7)	++			+	+	++
S/RCA-I	(3 b–4)	+			+	+	
	(5 a–5 b)		+	+	+	+	
	(6–7)		+	+	+	+	–
D/RCA-I	(3 b–4)	+			+	–	
	(5 a–5 b)		+	+	+	–	
	(6–7)		+	+	+	–	–
D/S/RCA-I	(3 b–4)	++			+	+	
	(5 a–5 b)		+	+	+	+	
	(6–7)		+	++	+	+	++
D/S/GSA-IB4	(3 b–4)	+			–	–	
	(5 a–5 b)	+			–	–	
	(6–7)	+			–	–	–
WGA	(3 b–4)	+++			++	++	
	(5 a–5 b)		+++	+++	++	++	
	(6–7)		+++	+++	++	++	+
S/WGA	(3 b–4)	+++			++	++	
	(5 a–5 b)		+++	+++	++	++	
	(6–7)		+++	+++	++	+[4]	+

[1] (+) and (–) indicate staining intensity on a semi-quantitative scale: (–) negative, (+) weak, (++) moderate and (+++) strong reaction; [2] S = Sialidase digestion, D = Desulphation; [3] Zona pellucida is stained either completely (uniform) or the internal or external part is stained; [4] Cumulus oophorus: ++

Table 4. Lectin positivity of feline preantral and antral follicles[1]

Lectin and treatment[2]	Follicular stage	Zona pellucida			Ooplasm	Granulosa cells	Follicular fluid and intercellular matrix
		Uniform	Internal	External			
S/SBA	(3 b–4)	+			–	–	
	(5 a–5 b)	+			–	–	
	(6–7)	+			–	–	–
D/SBA	(3 b–4)	+++			+	+	
	(5 a–5 b)	+++			+	+	
	(6–7)		+++	+++	+	+	+++
D/S/SBA	(3 b–4)	+++			+	+	
	(5 a–5 b)	+++			+	+	
	(6–7)	+++			+	+	+++
S/PNA	(3 b–4)	–	–	–	–	–	
	(5 a–5 b)	–	–	–	–	–	
	(6–7)	–	–	–	–	–	++
D/PNA	(3 b–4)	++			–	–	
	(5 a–5 b)	++			–	–	
	(6–7)	++			–	–	+
D/S/PNA	(3 b–4)	+++			+	+	
	(5 a–5 b)	+++			+	+	
	(6–7)	+++			+	+	+++
S/RCA-I	(3 b–4)	+			–	–	
	(5 a–5 b)	+			–	–	
	(6–7)		+	–	–	–	–
KOH/S/RCA-I	(3 b–4)	++			–	–	
	(5 a–5 b)	++			–	–	
	(6–7)	++			–	–	++
D/RCA-I	(3 b–4)	++			+	+	
	(5 a–5 b)	++			+	+	
	(6–7)	++			+	+	++
D/GSA-IB4	(3 b–4)	++			–	–	
	(5 a–5 b)	++			–	–	
	(6–7)	++			–	–	+
WGA	(3 b–4)	+++			++	++	
	(5 a–5 b)		+++	+++	++	++	
	(6–7)		+++	+++	++	++	+
S/WGA	(3 b–4)	+++			++	++	
	(5 a–5 b)		+++	+++	++	++	
	(6–7)		+++	+++	++	++	+

[1] (+) and (–) indicate staining intensity on a semi-quantitative scale: (–) negative, (+) weak, (++) moderate and (+++) strong reaction; [2] S = Sialidase digestion; [3] Zona pellucida is stained either completely (uniform) or the internal or external part is stained; [4] D = Desulphation.

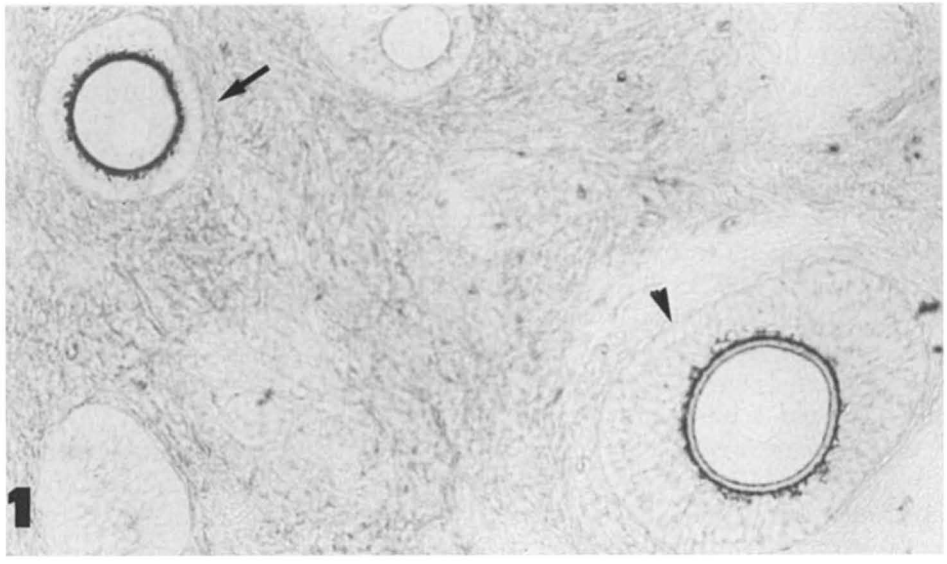

Fig. 1. Canine preantral follicles after neuraminidase treatment and SBA-HRP staining. After cleavage of sialic acids, SBA binding sites were present in large numbers and evenly distributed over the ZP of oocytes in stage 4 follicles (arrow) and were strongly stained in the outer and weakly stained in the inner part of the ZP of oocytes in stage 5 b follicles (arrowhead). Granulosa cells and ooplasm were negative. Magnification, ×230.

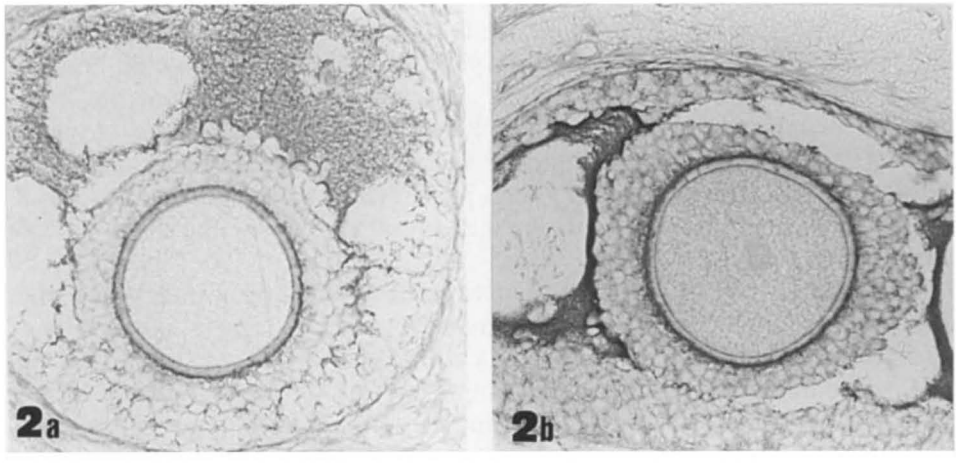

Fig. 2. Canine antral follicles in stage 7 after desulphation and SBA-HRP staining (a) and combined desulphation and neuraminidase treatment and SBA-HRP staining (b). Desulphation alone induced weak SBA positivity in the internal and external side of the ZP; granulosa cells and ooplasm were negative whereas follicular fluid/intercellular matrix were moderately positive. Neuraminidase digestion after desulphation caused an increase in SBA reactivity in the outer layer of the ZP, granulosa cells, ooplasm and in follicular fluid/intercellular matrix. Magnification, ×400.

and cat follicles. In both species, the ZP and follicular fluid/intercellular matrix strongly reacted with PAS and HID and reacted moderately with LID. Ooplasm and granulosa cells were always negative. Digestion with testicular and bacterial hyaluronidase, chondroitinase ABC, chondroitinase AC, chondroitinase B and heparitinase did not alter the intensity of both HID and LID staining of the ZP of oocytes in preantral and antral follicles. Positivity of follicular fluid and intercellular matrix was affected by enzymatic digestion. Treatment with testicular hyaluronidase reduced HID staining markedly and abolished LID staining completely, whereas bacterial hyaluronidase did not modify HID and LID reactivity of follicular fluid and intercellular matrix. Chondroitinase ABC, chondroitinase AC and chondroitinase B treatment notably diminished HID and LID positivity; whereas heparitinase treatment caused a slight decrease in HID staining.

Lectin histochemistry. In dog and cat, ZP showed positivity after staining with SBA, PNA, RCA-I, GSA-IB4 lectins but only after neuraminidase digestion and/or desulphation. Particularly in dog, removal of sialyl residues caused strong SBA staining which was uniform in the ZP of oocytes in follicles of stages 3 b–4 (Fig. 1) and was localised in the outer layer of the ZP of oocytes of stages 5 a–7; in the latter oocytes, only the inner part of the ZP was weakly labelled (Fig. 1). Desulphation induced weak SBA positivity which was uniformly distributed over the ZP of stages 3 b–4 oocytes and was localised in the internal and external surfaces of the ZP of oocyte in stage 5 a–7 follicles (Fig. 2 a). Labelling of SBA after desulphation and neuraminidase treatment was similar to that after neuraminidase digestion alone (Fig. 2 b). In cat, cleavage of sialic acids resulted in weak and homogeneous SBA positivity in the ZP of all oocytes examined. Desulphation induced strong staining with this lectin which was evenly distributed over the ZP of preantral oocytes in stages 3 b–5 b follicles, whereas staining was strong in the outer and inner boundary of the ZP and moderate in the central part of the ZP of antral oocytes (stages 6–7) (Fig. 3); enzymatic degradation after desulphation enhanced SBA positivity in the central portion of the latter type of oocytes (Fig. 4).

In dog, a uniform distribution pattern of PNA staining was detected after desulphation and neuraminidase treatment: it was strong in preantral and moderate in antral oocytes (Fig. 5). In cat, the amount of reaction product of PNA was moderate in preantral and antral oocytes following removal of sulphate groups; combined desulphation and neuraminidase treatment resulted in an increase of binding sites in the ZP of oocytes mentioned above (Fig. 6).

In canine ZP, few RCA-I binding-sites were found after neuraminidase digestion: they were uniformly distributed in the ZP of the oocytes of stages 3 b–4, whereas they were located in the outer and inner parts of the oocytes of stages 5 a–7 (Fig. 7); the same reaction pattern was also observed after desulphation. Desulphation and neuraminidase treatment resulted in an increase in RCA-I labeling in the oocytes of stages 3 b–4 and in the outer part of oocytes of stages 6–7 (Fig. 8). In cat, this lectin bound weakly and evenly to

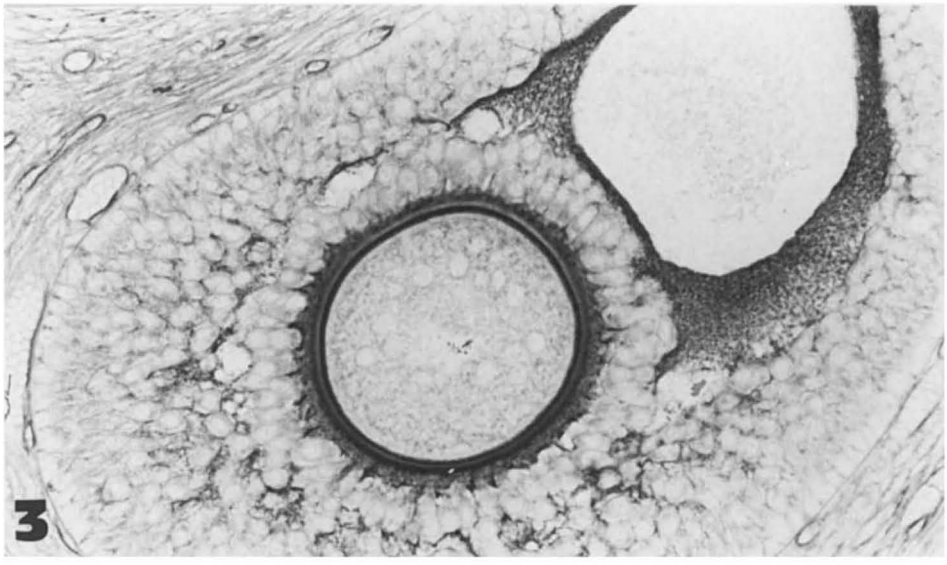

Fig. 3. Feline antral follicle (stage 7) after desulphation and SBA-HRP staining. After elimination of sulphate groups, SBA strongly stained the outer and inner parts of the ZP. Granulosa cells and ooplasm reacted weakly whereas follicular fluid and intercellular matrix reacted strongly. Magnification, ×400.

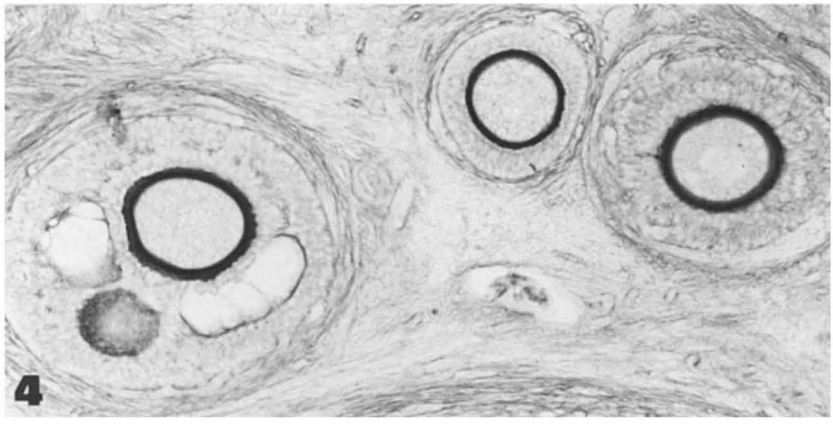

Fig. 4. Feline preantral (stages 4 and 5 a) and antral follicles (stage 6) after desulphation, neuraminidase treatment and SBA-HRP staining. Desulphation and subsequent neuraminidase treatment promoted uniform and strong SBA reactivity in the ZP of all follicles examined. Magnification, ×200.

the ZP of preantral oocytes and only to the internal part in antral oocytes after the cleavage of sialic acids; saponification moderately enhanced RCA-I staining which became uniform in the ZP of antral oocytes. Desulphation always resulted in a moderate and homogeneous reaction over the ZP, which was not modified by sialic acid degradation (Fig. 9).

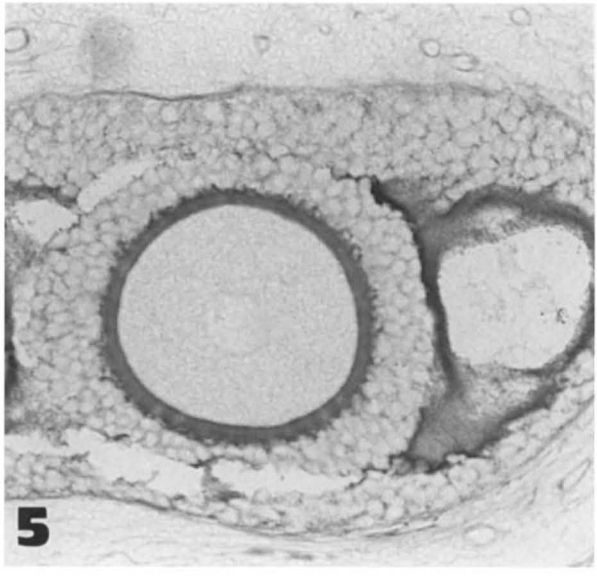

Fig. 5. Canine antral follicle (stage 7) after desulphation, neuraminidase treatment and PNA-HRP staining. PNA reactive sites were evenly distributed in a moderate way in the ZP; granulosa cells and ooplasm were weakly stained whereas follicular fluid and intercellular matrix were moderately stained. Magnification, ×400.

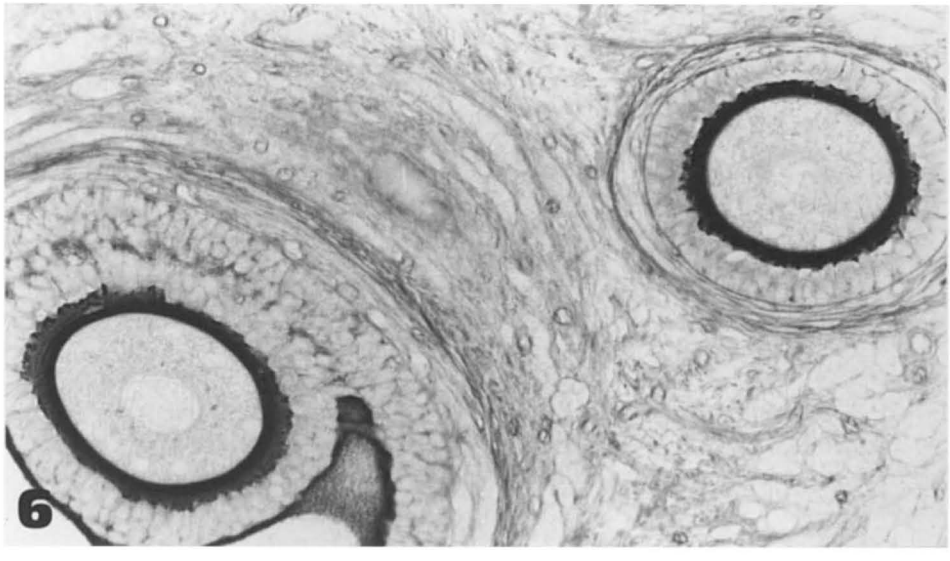

Fig. 6. Feline preantral (stage 4) and antral follicles (stage 7) after desulphation, neuraminidase treatment and PNA-HRP staining. After combined desulphation and neuraminidase treatment the reaction product of PNA was uniformly distributed in an abundant way in the ZP; granulosa cells and ooplasm showed a weak reaction whereas follicular fluid/intercellular matrix was strongly stained. Magnification, ×350.

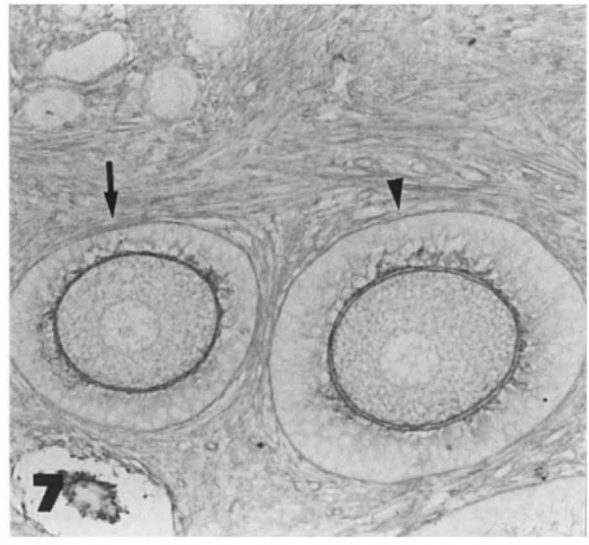

Fig. 7. Canine preantral follicles after neuraminidase treatment and RCA-I-HRP staining. Removal of sialyl residues resulted in weak uniform reaction over the ZP of oocytes of stage 4 (arrow) whereas it is localised in the outer and inner regions of the ZP of oocytes of stage 5 a (arrowhead); granulosa cells and ooplasm reacted weakly. Magnification, ×280.

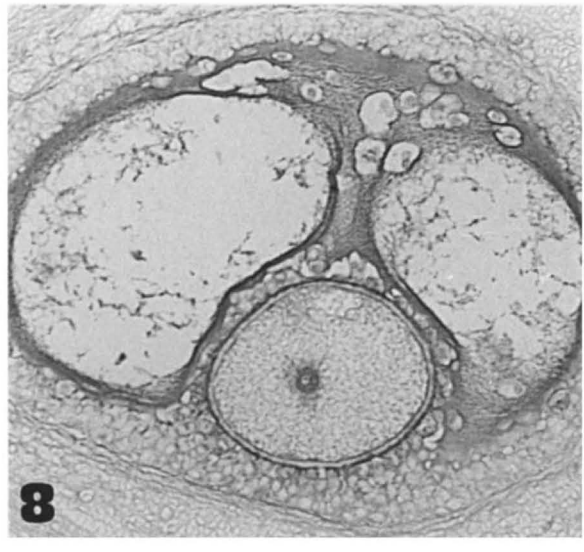

Fig. 8. Canine antral follicle (stage 7) after desulphation, neuraminidase treatment and RCA-I-HRP staining. ZP showed positivity that was moderate in the external part and weak in the internal part; granulosa cells and ooplasm exhibited a weak reaction whereas that of the follicular fluid/intercellular matrix was moderate. Magnification, ×280.

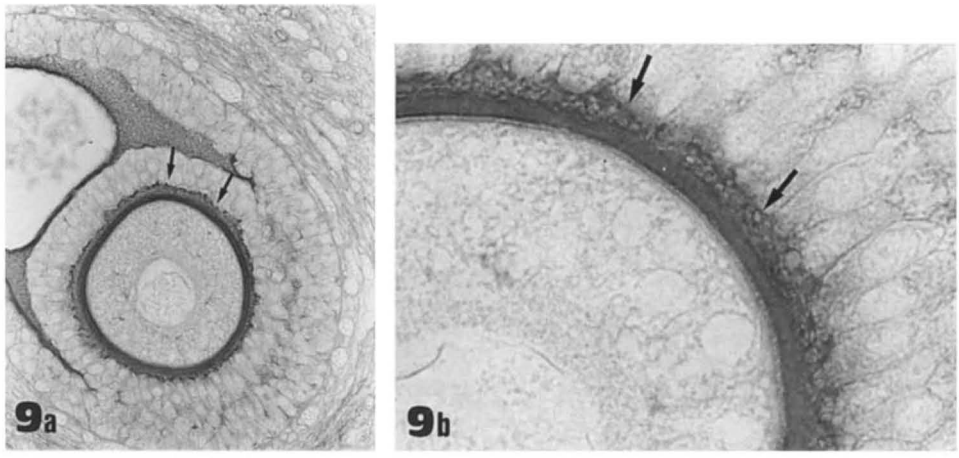

Fig. 9. Feline antral follicle (stage 7) after desulphation and RCA-I-HRP staining. a) The elimination of sulphate groups resulted in a moderate and homogeneous reaction; positivity was weak in granulosa cells and ooplasm and moderate in the follicular fluid and intercellular matrix. Magnification, ×380. b) Higher magnification of a): note the moderate reactivity of the zona material (arrows). Magnification, ×1 600.

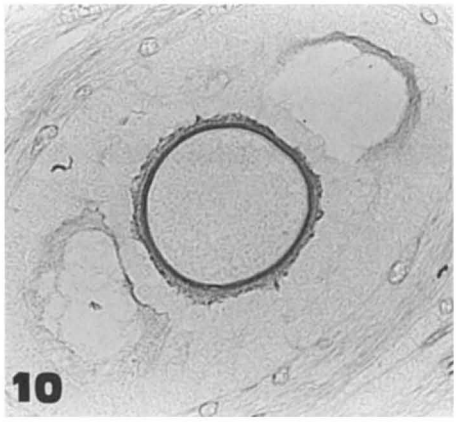

Fig. 10. Feline antral follicle (stage 6) after desulphation, neuraminidase treatment and GSA-IB4-HRP staining. GSA-IB4 staining was moderate and evenly distributed; granulosa cells and ooplasm were unstained whereas follicular fluid and intercellular matrix exhibited a weak reaction. Magnification, ×450.

GSA IB4-lectin stained the ZP of preantral and antral canine oocytes weakly and uniformly only after desulphation and neuraminidase treatment. In feline ZP, GSA-IB4-positive sites were moderately and evenly stained after desulphation; subsequent neuraminidase treatment did not enhance reactivity to this lectin (Fig. 10).

No differences in the labelling pattern were observed between canine and feline ZP when using WGA-lectin: reaction product was strongly and evenly distributed over the entire ZP in the oocytes of stages 3 b–4 whereas internal and external parts of oocytes of stages 5 a–7 were strongly stained. Sialic acid digestion with and without prior saponification and desulphation did not modify WGA-positivity (Fig. 11).

GSA-II, Con-A, LCA, UEA-I, LTA and DBA lectins always gave negative results in canine and feline ZP.

Ooplasm and granulosa cells exhibited very similar binding-patterns in dog and cat; these structures were moderately positive after staining with GSA-II, Con-A, LCA, UEA-I, LTA and WGA in both preantral and antral follicles. Additionally, a weak reaction was observed with SBA after desulphation in cat (Fig. 3), after desulphation and neuraminidase treatment in dog (Fig. 2 b) and with PNA after desulphation and neuraminidase treatment (Figs. 5, 6) in both canine and feline oocytes. RCA-I exhibited weak positivity after removal of sialic acids in canine oocytes (Fig. 7). Desulphation promoted weak staining only at the ooplasm level in dog and in both ooplasm and granulosa cells in cat (Fig. 9). GSA-IB4 staining was always negative in ooplasm and granulosa cells of both species (Fig. 10). A particular binding pattern was observed after staining with WGA lectin in dog. Granulosa cells of antral follicles showed a decrease in reactivity after degradation of sialic acids with the exception of the cumulus oophorus (Fig. 11).

In both species follicular fluid and intercellular matrix always showed a moderate reaction with GSA-II, Con-A, LCA, UEA-I, LTA and a weak reaction with WGA. After desulphation, SBA-reactive sites were moderately positive in dog (Fig. 2 a) and strongly positive in cat (Fig. 3); desulphation and neuraminidase treatment increased positivity in dog (Fig. 2 b). Sialic acid digestion and PNA staining resulted in moderate positivity in both species. In cat, desulphation caused a weak reactivity which strongly increased after neuraminidase treatment (Fig. 6). RCA-I binding sites were identified only after desulphation and neuraminidase treatment in dog (Fig. 8) and after neuraminidase treatment preceded by saponification or desulphation in cat. GSA IB4 gave a weak reaction after desulphation in cat. DBA failed to stain ooplasm, granulosa cells and follicular fluid and intercellular matrix in preantral and antral follicles of these animals.

Controls. When the lectin-HRP conjugates were preincubated with the appropriate hapten sugars or when they were omitted from the incubation medium, staining was not observed (Fig. 12). Desulphation abolished HID-positivity in control sections.

Discussion

Conventional histochemical staining methods indicated the presence of neutral and sulphated glycoconjugates in the ZP and follicular fluid whereas carboxylated radicals did not seem to be present. Enzymatic digestions that specifically remove glycosaminoglycans (GAGs) demonstrated the presence of large amounts of chondroitin sulphates B and A and/or C in canine and feline follicular fluid whereas the concentration of heparan sulphate was lower and hyaluronic acid was not present. These data are in agreement with previous studies in buffalo (Parillo et al., 1998), rat (Ax and Ryan, 1979), pig (Yana-

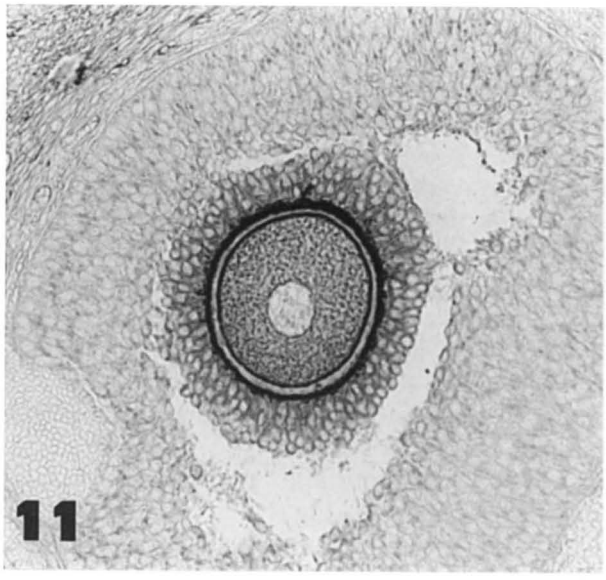

Fig. 11. Canine antral follicle (stage 7) after neuraminidase treatment and WGA-HRP staining. The labelling sites of this lectin were distributed in large amounts in the inner and outer parts of the ZP; granulosa cells were weakly stained whereas cumulus oophorus and ooplasm reacted moderately. Magnification, ×250.

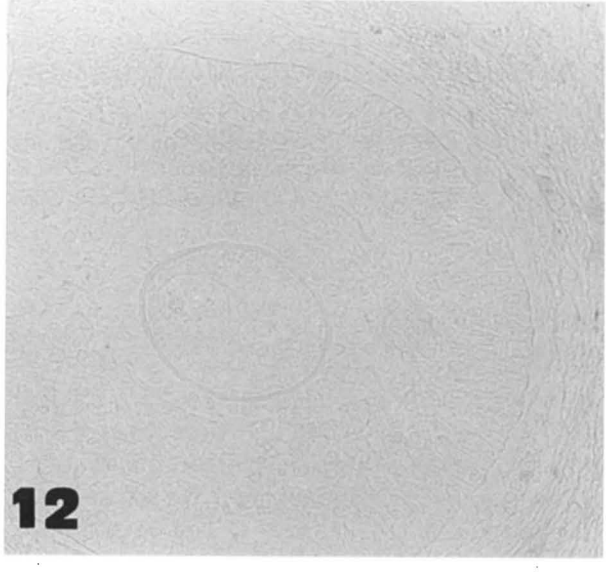

Fig. 12. Canine antral follicle (stage 6) after SBA-HRP staining in the presence of 0.2 M D-GalNAc. Staining was completely inhibited. Magnification, ×250.

gishita et al., 1979) and bovine (Lenz et al., 1982; Grimek et al., 1984; Bellin and Ax, 1987). The failure to digest GAGs in the ZP of both species confirms previous findings that hyaluronic acid (Dunbar et al., 1980; Roux and Kan, 1991) and other GAGs (Parillo et al., 1998) are not present in ZP. Lectin staining showed the presence of sulphated and non-sulphated sugars in the terminal and/or preterminal position, in particular β-D-GalNAc, D-Gal, α-D-Glc and/or D-Man, α-L-fuc and GlcNAc residues in the follicular fluid of both species.

Lectin histochemistry allowed us to better characterise glycoprotein material in follicles and to define the linkage type existing between the oligosaccharide chains and the polypeptides which constitute the canine and feline ZP. In analogy to other domestic species previously studied (Parillo et al., 1994a, 1996, 1998; Verini Supplizi et al., 1996) the asparagine and serine/threonine linked oligosaccharides were identified in the ZP of oocytes of dog and cat. In addition, we observed differences in lectin-binding affinity depending on species and stage of follicular development. Of particular interest was the labelling of the ZP of preantral and/or antral oocytes of both species with some lectins only after sequential methylation-saponification treatment, suggesting that D-GalNAc and α-and β-Gal residues may contain sulphated groups (Carter et al., 1988; Martinez-Menarguez et al., 1992; Avilès et al., 1994) and that they occupy a terminal position in the oligosaccharide side chains although these carbohydrates are typically internal residues in mammalian glycoconjugates. Staining and/or increase of staining with some of the other lectins after desulphation and neuraminidase treatment indicated that these sulphocarbohydrates also acted as receptor sugars for sialic acids. In contrast, GalNAc and Gal residues that were exposed only by sialic acid degradation, lacked sulfate esters which impede the binding capacity of some lectins (Stoward et al., 1980). In particular, mainly the disaccharide sialic acid-D-GalNAc, and to a lesser extent terminal sulpho-D-GalNAc were detected in canine ZP of preantral and antral oocytes as was shown by neuraminidase treatment or desulphation and subsequent SBA staining, respectively. In feline oocytes, few D-GalNAc residues were found to be linked with sialic acid; sulpho-D-GalNAc was evenly distributed in the terminal position in preantral oocytes whereas labelling of this sugar was unevenly distributed in antral oocytes, showing a higher concentration in the outer and inner parts of the ZP and a lower concentration in the central part where it was also linked with sialyl residues.

In all cases, D-GalNAc residues, with or without sulphated esters, were found in the β-anomeric linkage since staining with DBA lectin was always negative.

Numerous β-Gal residues were found in the canine and feline ZP as revealed by the large amounts of binding sites for PNA and RCA-I lectins, which differ according to their recognition of the terminal dimer. The former lectin specifically recognises the sequence β-Gal-(1–3)-D-GalNAc and the latter lectin the sequence β-Gal-(1–4)-GlcNAc. In the ZP of all oocytes of both

species that have been examined, we observed that the β-Gal linked to GalNAc always contained sulphated groups since desulphation was required to establish effective labelling of PNA. In particular, β-sulfoGal-(1–3)-D-GalNAc was shown only to be linked with sialic acids in dog and in terminal and subterminal positions in cat. In contrast, β-Gal linked to GlcNAc was identified in both sulphated and non-sulphated forms. The former was found in the terminal position in all oocytes of both species and linked to sialic acids in canine oocytes; the latter was detected in ZP of both species subterminal to sialic acids which also contained C4 O-acetyl substituents, but only in cat.

α-Gal residues also contained sulphated esters: they were detected to be linked to sialic acid in canine oocytes, as revealed by positivity after desulphation, neuraminidase treatment and GSA-IB4 staining, and in the terminal position in feline oocytes since enzymatic degradation did not modify desulphation and GSA-IB4 staining.

Positivity after WGA lectin staining, which was similar in the ZP of both species, and negativity after GSA-II lectin staining suggests that GlcNAc residues were present in the internal position of the oligosaccharide chains. In addition, sialic acid did not compete with GlcNAc for WGA at this level (Monsigny et al., 1980) since sialic acid digestion, also after deacetylation with KOH, did not modify the intensity or distribution of staining with this lectin.

Sialic acids were not revealed by LID staining, but sialoglycoconjugates having sialic acid (α-2–3,6)-α- and -β-Gal and sialic acid (α-2–6)-β-D-GalNAc as terminal sequences were demonstrated after neuraminidase treatment and GSA IB4/PNA/RCA-I/SBA staining. The presence of sialic acids, acetylated or not, has also been demonstrated in mouse (Tadano and Yamada, 1978), hamster (Delgado and Zoller, 1987), man (Bar-Shira Maymon et al., 1994) and livestock (Parillo et al., 1994b, 1996, 1998; Verini-Supplizi et al., 1996). Because sialic acids are negatively charged, they are involved in binding and transport of positively charged compounds which play a role in hydration of the ZP. Sialic acids also influence the conformation of glycoproteins which act as receptor sites for sperm cells, keeping these glycoproteins inactive until fertilisation takes place. In addition, the rigidly restricted α-2–3 linkage in conjuction with the more freely rotating α-2–6 bond gives viscoelastic properties to the ZP which may facilitate penetration of sperm cells during fertilisation.

Numerous sulphated groups were detected in both canine and feline ZP. The importance of sulphated esters in making specialised biological activities of carbohydrates more effective is well documented. Biochemical and histochemical studies (Shimizu et al., 1983; Mori et al., 1991; Noguchi et al., 1992; Avilès et al., 1994) have demonstrated that glycoproteins of mouse and pig ZP are sulphated in their oligosaccharides, some of which are involved in sperm-egg interactions. Additionally, some authors (Se Gall and Lenarz, 1979; Kopf and Garbers, 1980; Rosignol et al., 1984; De Angelis and Glabe, 1988) have suggested that in Echinoidea spp. sulphoglycoconjugates on the

sperm membrane induces acrosome reactions in the presence of calcium and that sulphation of the sperm receptor is necessary for egg receptor complementarity.

Our results indicate differences in the carbohydrate residue content, and in their spatial distribution between the ZP of preantral and antral oocytes of both species. In particular, the sugar residues were homogeneously distributed throughout the entire ZP at early stages (3 b–4) of oocytes development. As the follicles mature, ZP glycoproteins continue to be deposited and the addition of newly synthesised sites that react with lectins tends to be uneven. This asymmetric arrangement of lectin binding sites into two or more distinct bands has also been observed in other species (Shalgi et al., 1991; Skutelsky et al., 1994; Parillo et al., 1996, 1998; Verini Supplizi et al., 1996), indicating differences in the presence, density and distribution of glycoproteins within the ZP. Ahuja and Bolwell (1983) suggested that the immunological dissimilarity between the inside and outside of the hamster ZP may be related to the unequal distribution of sperm receptors within the ZP itself.

Ooplasm and granulosa cells exhibited a very similar lectin-profile in both species. Carbohydrate residues, such as β-D-GalNAc, D-Gal, α-D-Glc and/or D-Man, α-L-fuc and GlcNAc, were not demonstrated by conventional methods, but were revealed by lectins.

In canine follicles, neuraminidase treatment induced a decrease of WGA staining of granulosa cells, but not of the cumulus oophorus and ooplasm, indicating the presence of large amounts of sialic acids (Monsigny et al., 1980). The presence of the disaccharide sialic acid-GlcNAc can be ruled out since neuraminidase treatment failed to modify GSA-II reactivity.

In both species, we have demonstrated the presence of ZP material between granulosa cells and the compact layers of the ZP as was previously observed in dog by Tesoriero (1984) using conventional histochemical methods and ultrastructural cytochemistry. Tesoriero suggested that both the oocyte and its follicle cells participate in ZP synthesis as demonstrated by studies carried out in other animals (Gwatkin et al., 1979; Wolgemuth et al., 1982). The similarity in lectin affinity between ooplasm and ZP of oocytes present in follicles at different stages of development seems to confirm the involvement of oocytes in ZP production. The application of immunocytochemical techniques associated with ultrastructural investigations could elucidate the role of ooplasm in the ZP synthesis in these species.

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