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Glycocomposition of the apocrine interdigital gland secretions in the fallow deer (*Dama dama*)

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ABSTRACT

The secretions of the tubular interdigital glands were investigated by conventional (Periodic-Acid Schiff, Alcian-Blue at different pH, Low Iron Diamine and High Iron Diamine) and lectin (Con-A, UEA-I, LTA, WGA, GSA-II, GSA-IB4, SBA, PNA, ECA, DBA, MAL-II and SNA) histochemical methods in adult males and females of different age of fallow deer during the breeding season. Sialidase digestion and deacetylation pre-treatment were also employed in conjunction with lectin histochemistry.

The glandular epithelium consisted of a single layer of low columnar cells with typical apical protrusions. No substantial differences of the above histochemical staining in relation to sex and age were observed. Conventional histochemical staining revealed that the interdigital glands secreted neutral glycoproteins whereas acidic glycocomponents did not seem to be present. Lectin histochemical technique allowed us to disclose a great heterogeneity of glycoproteins with *N*- and *O*-linked oligosaccharides containing α-D-Man/α-D-Glc, GlcNAc, α-Fuc, terminal β-D-Gal-(1-3)-D-GalNAc, -D-Gal-(1-4)-D-GlcNAc, α-Gal and β-GalNAc residues. β-GalNAc and disaccharide β-D-Gal-(1-3)-D-GalNAc were also found as subterminal to sialyl moieties. The lack of sexual and age-related differences in the glucidic content of the glandular secretions seems to indicate that the glycoderivatives may play only an accessory role in the production of odoriferous signals in fallow deer.

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1. Introduction

In Italy, fallow deer (*Dama dama*) is the most common cervid kept in national parks, reserves and extensively managed farms (Randi, 2005). These cervids are seasonal breeders: the breeding season, known as the rut, takes place in the fall, beginning in mid-September and continuing into November, but the peak breeding activity takes place in October. Fallow deer populations show a variety of mating systems, ranging from low-fidelity territorial/follower strategy (Moore et al., 1995) to lekking and deer harems (Clutton-Brock et al., 1988), but variance in mating success is generally very high (Moore et al., 1995; Clutton-Brock et al., 1988; Apollonio et al., 1989). Females reach sexual maturity at 16 months whereas bucks are mature sexually at 14 months but rarely compete successfully in rutting until several years later (Chapman and Chapman, 1970, 1980).

As in other wild ungulates, the communication of reproductive information in fallow deer is thought to be accomplished by odours associated with skin glands localized to specific areas, such as infraorbital, tarsal and interdigital areas (Osborn et al., 2000). The interdigital glands belong to a group of skin apocrine glands, varied in morphology, described in several Artiodactyla, among them in various species of deer (Wood, 1999, 2003; Wood et al., 1995a, b; Reiter et al., 2003), in the Japanese serow, *Capricornis crispus* (Atoji et al., 1988), in the impala, *Aepyceros melampus* (Welsch et al., 1998) and in the Asian elephant, *Elephas maximus* (Lamps et al., 2001). As regards their function, these glands are considered scent glands producing odorous signals and pheromones that play important biological roles in the conspecific chemical communication, such as active territorial demarcation and in the expression of social behaviour (Robertshaw, 1987; Epple et al., 1993). Several studies have been carried out in order to identify the chemicals in the secretions of skin glands, including foot or interdigital glands of reindeer, *Rangifer tarandus* (Brundin et al., 1978; Andersson et al., 1979; Brundin and Andersson, 1979), white-tailed deer, *Odocoileus virginianus* (Gassett et al., 1996; Wood, 1999), and black-tailed deer, *Odocoileus hemionus columbianus* (Wood et al., 1995a, b), the forehead gland of white-tailed deer (Gassett et al., 1997), the tarsal gland of black-tailed deer (Müller-Schwarze et al., 1978) and reindeer (Andersson et al., 1975), the preorbital gland of reindeer (Sokolov et al., 1977; Andersson, 1979), metatarsal gland of sika deer, *Cervus nippon* (Wood, 2003), the caudal gland of reindeer (Müller-Schwarze et al., 1977), and the tail gland of red deer, *Cervus elaphus* (Bakke and Figenschou, 1983). Similarly to other odoriferous glands, the major constituents of the scent

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material secreted by the interdigital glands are volatile elements such as alkanes, isoalkanes, ketones and aldehydes, already biochemically characterized in a range of species (Reiter et al., 2003; Wood, 2003). However, among the secretory products of some scent glands, also non-volatile substances, such as glycoconjugates with different terminal sugars have been detected by means of lectin histochemical techniques (Atoji et al., 1988; Aoki-Komori et al., 1994; Welsch et al., 1998). Lectins are specific carbohydrate binding proteins of non-immune origin that agglutinate cells or precipitate polysaccharides or glycoconjugates, which have been widely used as histochemical probes for localizing and characterizing specific sugar residues or oligosaccharide sequences in cells and tissues (Spicer and Schulte, 1992).

Carbohydrates are involved in many biological activities and they are important key substances for the functional properties of the secretion of many exocrine glands (Damjanov, 1987; Spicer and Schulte, 1992).

To our knowledge, there is a lack of information on the glycocomposition of the secretory products of the fallow deer interdigital glands. In the present study, we first examined the morphology of the interdigital glands and then applied lectin histochemistry combined with enzymatic digestion and chemical treatments to it for analyzing the presence and distribution of the carbohydrate binding sites in these glands. In order to better understand some aspects of the biology of the fallow deer, possible sex- and age-related differences in the lectin affinity of the interdigital glands were also investigated.

2. Materials and methods

2.1. Animals

The whole interdigital glands of both fore and hind legs were collected from adult male ($n = 6$; aged from 18 months to 5-year-old) to adult female ($n = 6$; aged from 2 to 8-year-old) fallow deer during the breeding season (October–November). These skin samples were excised immediately after death of the animals, which were regularly slaughtered in an authorized abattoir for wild ungulates following principles of animal care and specific national laws. After gross dissecting, the interdigital glands were drawn and immediately fixed as follows.

2.2. Tissue preparation

The specimens of both fore and hind legs were immediately fixed in Carnoy's fluid for 24 h and post-fixed in a solution of 2% calcium acetate and 4% paraformaldehyde (1:1 v/v) for 3 h at room temperature (Menghi, 1984). They were then routinely dehydrated in graded series of alcohols, cleared in xylene and subsequently embedded in paraffin.

Serial sections 5 μm thick were mounted on Superfrost Plus slides (Bio-Optica, Milano, I) and subjected to conventional and lectin histochemical staining.

2.3. Conventional histochemical staining

The sections were stained with the following methods: Periodic-Acid Schiff (PAS) to detect vicinal hydroxyls, Alcian-Blue (AB) pH 2.5 and Low Iron Diamine (LID) to demonstrate acidic groups and AB pH 1.0, AB pH 0.5, and High Iron Diamine (HID) to discriminate sulphate groups (Pearse, 1985).

2.4. Lectin histochemistry

The specimens were processed for lectin histochemistry according to the procedures that were described previously by Parillo and Verini Supplizi (2008).

Table 2
Lectin binding patterns in the interdigital glands of fallow deer[a]

Staining and treatments	Glandular cells	Apical cytoplasmic protrusions
PAS	2	2
AB pH 2.5 and LID	0	0
AB pH 1 and HID	0	0
AB pH 0.5	0	0
Con-A	3	3
LTA	3	3
UEA-I	2	3
WGA	1	2
Sialidase/WGA	1	2
PNA	1	2
Sialidase/PNA	2	3
SBA	1	1
Sialidase/SBA	2	2
ECA	1	2
Sialidase/ECA	1	2
GSAIB4	1	2
Sialidase/GSAIB4	1	2
DBA	0	0
Sialidase/DBA	0	0
GSA-II	0	0
Sialidase/GSA-II	0	0
SNA	1	2
MAL-II	1	2

[a] Evaluations were performed by attributing scores from 0 to 3 according to the following criteria: the reactivity was classified as absent (score of 0) when there are non-glandular cells or apical protrusions per high-power field (HPF, using 400× magnification), weak (score of 1) for a few cells (1–19) per HPF, moderate for a discrete number of cells (20–49) per HPF, and strong (score of 3) for numerous cells (50–99) per HPF.

Table 1
Lectins used and their carbohydrate specificities

Source of lectin	Acronym	Carbohydrate specificity[a]	Inhibitory sugars[b]	Lectin concentration (μg/ml)
Canavalia ensiformis	Con-A	α-D-Man > α-D-Glc	α-D-Methylman	20
Triticum vulgaris	WGA	GlcNAc > sialic acid	D-GlcNAc	10
Griffonia simplicifolia	GSA-II	α and β GlcNAc	D-GlcNAc	50
Glycine max	SBA	α-D-GalNAc > β-D-GalNAc	D-GalNAc	10
Arachis hypogaea	PNA	β-D-Gal-(1 → 3)-D-GalNAc	D-Gal	40
G. simplicifolia IB4	GSA-IB4	α-D-Gal	D-Gal	20
Ricinus communis	ECA	β-D-Gal-(1 → 4)-D-GlcNAc	D-Gal	50
Dolichos biflorus	DBA	α-D-GalNAc	D-GalNAc	10
Lotus tetragonolobus	LTA	α-L-Fuc	L-Fuc	20
Ulex europaeus	UEA-I	α-L-Fuc	L-Fuc	20
Sambucus nigra	SNA	NeuAc(α2,6)Gal/GalNAc	NeuAc	100
Maackia amurensis	MAL-II	NeuAc(α2,3)Gal	NeuAc	100

[a] β-D-Gal, β-D-galactose; α-D-Gal, α-D-galactose; D-GalNAc, D-*N*-acetylgalactosamine; β-D-GalNAc, β-D-*N*-acetylgalactosamine; α-D-GalNAc, α-D-*N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; α-D-Man, α-D-mannose; α-D-Glc, α-D-glucose NeuAc, *N*-acetylneuraminic acid.
[b] α-D-Methylman, α-D-methylmannose.

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The sections were dipped in 0.3% H_2O_2/methanol for 30 min to inhibit endogenous peroxidase activity and, after washing with PBS, were incubated in a moist chamber for 1 h at room temperature with a solution of horseradish peroxidase (HRP) conjugated lectins in 0.1 M PBS pH 7.2 containing 0.1 mM $CaCl_2$, $MgCl_2$ and $MnCl_2$. The sections were gently rinsed with PBS and the peroxidase activity sites were visualized with a 3-3′ diaminobenzidine (DAB kit, D.B.A. Italia S.R.L., Milano, I) for 5 min. Finally, all the sections were rinsed in distilled water, dehydrated and mounted in Eukitt.

2.5. Biotinylated lectins specific for sialic acid

The direct visualization of sialic acid residues was carried out with SNA and MAL-II biotinylated lectins. Sections were dipped in 0.3% H_2O_2/methanol for 30 min to inactivate endogenous peroxidase. Endogenous avidin-binding activity was blocked using the avidin–biotin blocking kit (D.B.A. Italia S.R.L., Milano, I). Subsequently, the sections were processed as above with the exception that, after lectin incubation, the slides were exposed to avidin–biotin complex (ABC kit, Vector Laboratories) for 30 min.

All the lectins used in this research, as well as their carbohydrate specific, inhibitory sugars, and optimal concentrations, are reported in Table 1. The lectins were purchased from Sigma Chemicals (Milano, Italy) with the exception of GSA-II, LTA, ECA, MAL-II and SNA that were obtained from Società Italiana Chimici (Roma, I).

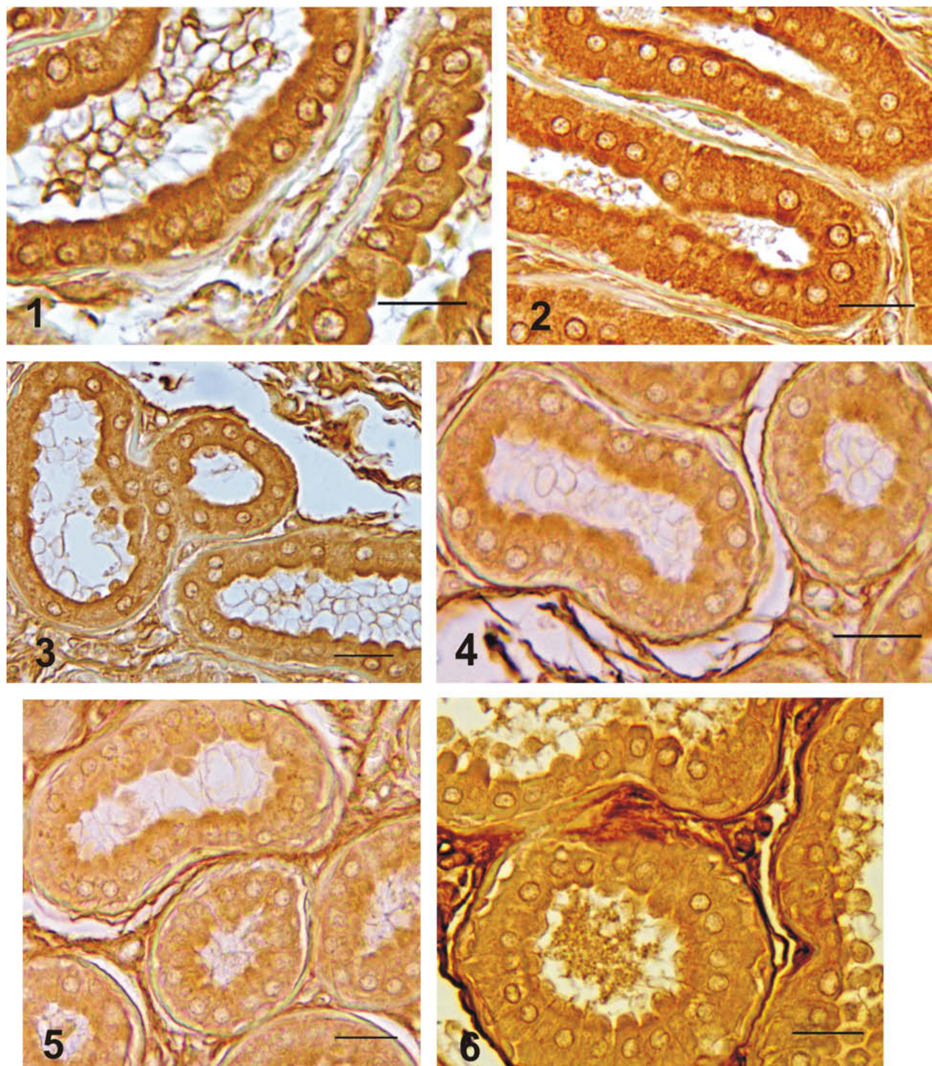
2.6. Controls

Negative controls for the lectin labelling, except MAL-II and SNA, were run either by substitution of lectin-conjugates with the respective unconjugated lectins or by preincubation of lectins with the addition of 0.2/0.4 M corresponding hapten sugars (Sigma, Milano, Italy). Preincubation of sections with neuraminidase eliminated staining with MAL-II and SNA.

3. Results

3.1. Anatomy and histology

The interdigital glands were located between the digits of the fore and hind-feet. Histologically, they consisted of a superficial



Figs. 1–6. Lectin histochemistry of the male and female fallow deer interdigital glands. (1) Male 5-year-old, Con-A binding. The lectin strongly stains the glandular cells and the apical cytoplasmic protrusions. Bar = 10 μm. (2) Female 7-year-old, LTA binding. The glandular cells and the apical protrusions strongly react. Bar = 10 μm. (3) Male 5-year-old, UEA-I binding. The reactive sites are moderately localized in the glandular cells and strongly in the apical protrusions. Bar = 20 μm. (4) Male 3-year-old, WGA binding. The lectin weakly stains the glandular cells and moderately the apical protrusions. Bar = 10 μm. (5) Female 2-year-old, PNA binding. The labelling sites are weakly localized in the glandular cells and moderately in the apical protrusions. Bar = 10 μm. (6) Female 2-year-old, sialidase/PNA sequence. After the cleavage of sialic acid residues PNA binding sites are moderately localized in the glandular cells and strongly in the apical protrusions. Bar = 10 μm.

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layer of sebaceous glands located just beneath the skin and of a deeper layer of tubular glands, connected among them by hair follicles. In the present study, we focused our attention on the histochemical features of the tubular glands. These were arranged into lobules divided by fibro connective tissue. The glandular epithelium consisted of a single layer of low columnar cells with typical apical protrusions. There were no sex- and age-related differences in the morphology of these glands. Table 2 legend indicate the specific score attributed to each lectin histochemical analysis.

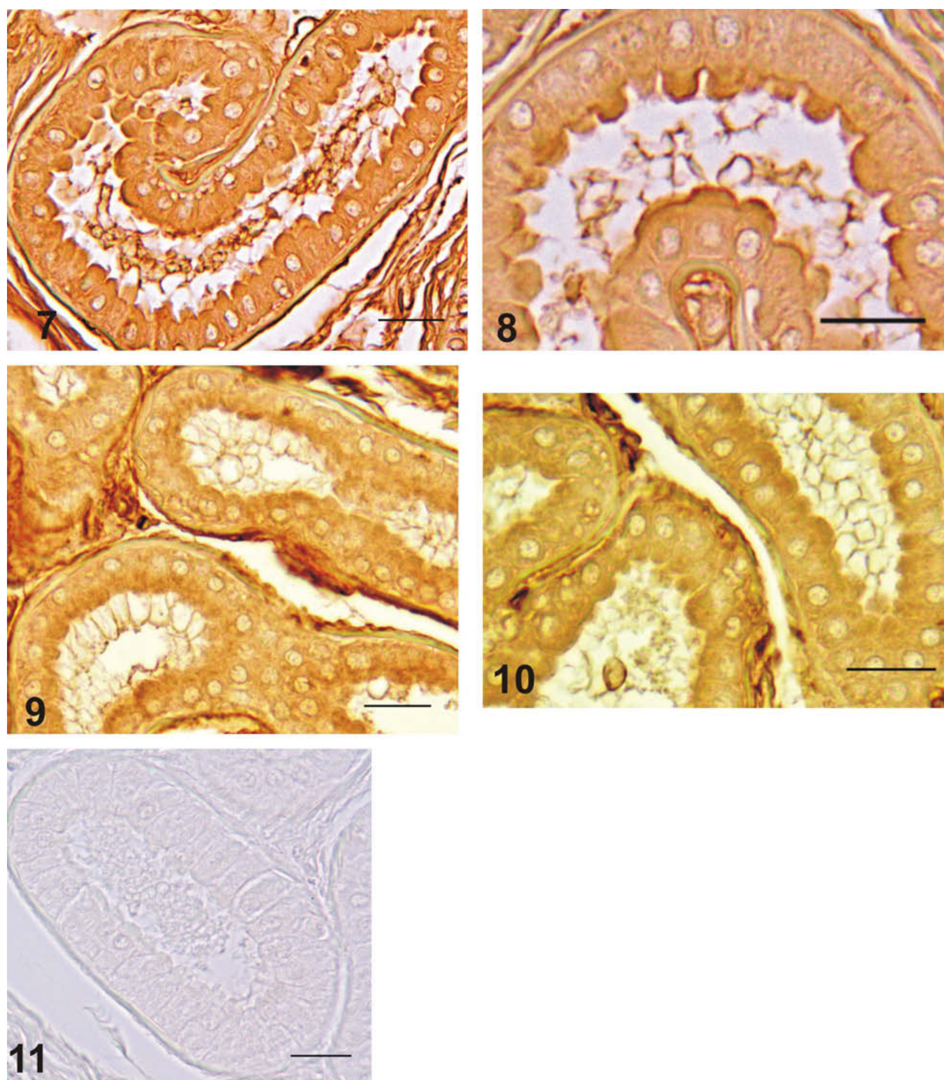
3.2. Histochemical composition

The conventional and lectin histochemical profiles of the fallow deer tubular glands are summarized in Table 2. No substantial differences of the above histochemical staining in relation to sex and age were observed. The PAS procedure moderately stained the entire cytoplasm of the glandular cells and the apical protrusions, whereas all the other conventional histochemical staining resulted negative in the histological sites considered. Lectin histochemistry revealed positive binding for Con-A, UEA-I, LTA, WGA, GSA-IB4, SBA, PNA, ECA, MAL-II and SNA.

In particular, Con-A and LTA stained strongly the cytoplasm of the glandular cells and the apical protrusions (Figs. 1 and 2).

UEA-I exhibited a moderate staining in the glandular cells and a strong reactivity of the apical protrusions (Fig. 3). WGA and PNA weakly stained the glandular cells and moderately the apical protrusions (Figs. 4 and 5); sialidase degradation did not change WGA labelling whereas it enhanced PNA positivity in both histological sites (Fig. 6). SBA weakly reacted in the glandular cells and apical protrusions; enzymatic digestion increased the positivity in the above sites (Fig. 7).

ECA, GSA-IB4 (Fig. 8), SNA (Fig. 9) and MAL-II (Fig. 10) weakly stained the glandular cells and moderately the apical cytoplasmic protrusions; sialidase digestion did not modify ECA- and GSA-IB4-binding sites. DBA- and GSA-II-lectins resulted constantly negative. Saponification with KOH never modified the results obtained with sialidase digestion, prior to lectin staining, in any of the histological sites studied.



Figs. 7–11. Lectin histochemistry of the male and female fallow deer interdigital glands. (7) Male 3-year-old, sialidase/SBA sequence. The enzymatic digestion determines a moderate SBA staining in the glandular cells and apical protrusions. Bar = 20 μm. (8) Male 5-year-old, GSA-IB4 binding. This lectin weakly reacts in the glandular cells and moderately in the apical cytoplasmic protrusions. Bar = 10 μm. (9) Male 2-year-old, SNA binding. The reactive sites are weakly localized in the glandular cells and moderately in the apical protrusions. Bar = 20 μm. (10) Female 7-year-old, MAL-II binding. The glandular cells weakly react whereas the apical protrusions moderately. Bar = 10 μm. (11) Female 4-year-old, control section. Con-A HRP with 0.4 M D-Man. Staining is completely inhibited. Bar = 10 μm.

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3.3. Staining controls

All the control staining procedures failed to disclose appreciable reactivity at all of the sites described in the fallow deer interdigital glands (Fig. 11).

4. Discussion

Conventional histochemical staining revealed that the interdigital tubular glands secreted neutral glycoproteins, containing hexoses with vicinal hydroxyls visualized with the PAS procedure. Acidic glycocomponents did not seem to be present as demonstrated by the negativity of AB pH 1.0, AB pH 2.5, AB pH 0.5, LID and HID staining.

More detailed information on the oligosaccharide side chains of the glycoproteins secreted by the interdigital glands was obtained by means of the lectin histochemistry. This high sensitive histochemical technique helped us to disclose a great heterogeneity of glycoproteins with *N*- and *O*-linked oligosaccharides, as demonstrated by the affinity of numerous lectins in the glandular cells and in the apical cytoplasmic protrusions. The conspicuous presence of these apical projections seems to suggest that the fallow deer interdigital glands secrete its products mainly in an apocrine manner. However, the coexistence of a merocrine secretion, as previously described in other scent glands can not be excluded (Smith and Hearn, 1979; Welsch et al., 1998).

In particular, Con-A and WGA labelling sites revealed the occurrence of α-D-Man/α-D-Glc and GlcNAc residues, respectively. These carbohydrates may be associated with the initial assembly of the *N*-linked oligosaccharides of glycoproteins (Chan and Wong, 1992). GlcNAc moieties showed to be present in the internal position of the oligosaccharide side chains, as demonstrated by the negativity of the GSA-II-lectin which, conversely, recognizes GlcNAc residues in terminal position. In this study the terminal disaccharide sialic acid-GlcNAc can be ruled out, because sialidase digestion failed to unmask GSA-II reactive sites.

LTA- and UEA-I-lectins with the same nominal specificity for α-fucose residues showed a different reactivity. Indeed, LTA marked strongly the glandular cells and the apical protrusions whereas UEA-I moderately. Similar discrepancies among lectins with the same nominal monosaccharide specificity have been already reported (Damjanov, 1987). In our study, the fucose secreted by the glandular cells was mainly α 1,4 bound to GlcNAc in *N*-linked oligosaccharides, whereas a minor amount was represented by α 1,2 bound to GalNAc in *O*-linked glycans (Spicer and Schulte, 1992). α-Fucose is the predominant terminal sugar in glycoproteins pertaining to membranes with a rapid turnover, such as those involved in secretion processes (Bennet et al., 1974).

The reactivity of PNA, ECA, GSA-IB4 and SBA indicated the presence of terminal β-D-Gal-(1-3)-D-GalNAc, β-D-Gal-(1-4)-D-GlcNAc, α-Gal and D-GalNAc residues, respectively. *N*-acetylgalactosamine moieties occurred in β-anomeric form as evidenced by SBA reactive sites and DBA negativity. Conventional histochemical staining did not reveal the occurrence of sialoderivatives (see LID and AB pH 2.5 negativity), but they were detected by lectin histochemistry.

Indeed, our results indicated that the glandular cells secreted Neu5Acα2,3Gal and Neu5Acα2,6Gal/GalNAc sugar sequences as evidenced by MAL-II and SNA positivity, respectively. Furthermore, the indirect visualization of sialic acid demonstrated that the acceptor sugars for sialyl residues were β-GalNAc and the disaccharide β-D-Gal-(1-3)-D-GalNAc as demonstrated by SBA and PNA reactivity after sialidase degradation.

Sialyl moieties did not seem to contain *O*-acetyl substituents at C4 since saponification with KOH did not change the staining obtained with sialidase/SBA and sialidase/PNA sequences.

Comparing these findings with those obtained from previous studies on the histochemical composition of the interdigital glands of other species (Atoji et al., 1988; Welsch et al., 1998), we evidenced some differences in the glucidic composition, i.e., Man/Glc and Fuc residues were not detected in the impala (*A. melampus*) whereas α-GalNAc moieties were identified in the Japanese serow (*C. crispus*) but not in the fallow deer. This seems to suggest the existence of a specie-specificity of the glycocomponents secreted by these skin glands.

The interdigital glands have been suggested to be scent glands marking in trails in red duiker (*Cephalophus natalensis*) (Ainoya, 1978; Langguth and Jackson, 1980) but not in the moose (*Alces alces*) (Chapman, 1985). Similarly, some authors doubted that these glands could play a role in active marking during locomotion in Japanese serow (Atoji et al., 1988). In our study, the lack of sexual and age-related differences in the glucidic content of the glandular secretions seems to indicate that glycoderivatives probably have only an ancillary role as scent substances in fallow deer. Conversely, in the impala species, the glands of the male forehead showed differences in the expression of some lectins suggesting the hypothesis that glycoconjugates secreted by these glands have a signalling role in the rut (Welsch et al., 1998).

In our study, we evidenced that the most abundant carbohydrates detected in the secretion of the interdigital glands are fucosyl and sialyl residues. The known hydrophobicity of fucose (Montreuil, 1980) and the high degree of viscosity conferred by sialic acids to the mucins (Schauer, 1978) could contribute to form a glue-like material in which the volatile substances, primary involved in olfactory communication, could be embedded. In our opinion, the glycoconjugates secreted by these glands could act as a long-lasting carrier of the volatile components. This hypothesis is supported by the chemical composition of the interdigital secretions of the red hartebeest (Reiter et al., 2003). The presence of many hydrophilic compounds as alcohol, ketones and carboxylic acid may suggest a possible formation of hydrogen bounds between the above substances and the hydroxyl residues of the glycoproteins secreted by the interdigital glands. In addition, the presence of sialic acids with the rigidly restricted α-2-3 linkage in conjunction with the more freely rotating α-2-6 bond could confer viscoelastic properties to the glue which may facilitate the gradual release of the volatile compounds.

In conclusion, on the basis of the present results we suggest that the interdigital glands of the fallow deer may play only an accessory role in the production of odoriferous signals in fallow deer. This hypothesis is further supported by the findings obtained in the white-tailed deer tarsal glands (Osborn et al., 2000) where no age, sex and season-related variation has been detected in the activity of the glandular tissues. The authors suggested that the odours on the tarsal gland may be ascribed from interactions among glandular secretions, urinary constituents and microbial decomposition.

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