Morphological Aspects and Expression of Estrogen and Progesterone Receptors in the Interdigital Sinus in Cyclic Ewes

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ABSTRACT Many species that belong to Artiodactyls order show an interdigital sinus (IS), as it occurs in sheep, in all four extremities. These are considered to be scent glands responsible for sexual communication having strong attractiveness to mature males at the peak of the breeding season. The aim of this study was to evaluate, in IS in cyclic ewes, the microscopic and ultra-structure (scanning and transmission electron microscopy) anatomy, secretion composition, and mRNA and protein expression of estrogen receptors α and β and progesterone receptors. Glandular sebaceous structures occupy a superficial area of the pouch. The other glands present in the IS show a coiled tubular structure and tall and polyhedral secretory cells with irregular luminal surface resulting from the secretory process. Protein and mRNA gene transcription studies were performed to determine the presence of ER (α and β) and P4r in IS. At the follicular phase, IS cell populations analyzed using flow cytometry expressed higher levels of ERα compared with ERβ (P < 0.05), whereas no difference was observed between them in the luteal phase. The IS amount of secretion was the highest in the follicular phase compared with luteal phase (P < 0.05) or pregnancy (P < 0.001). To the best of our knowledge, for the first time, the presence of ER (α and β) within the IS was demonstrated. As estrogen action is mediated by specific receptors in target cells, the presence of these receptors in IS might be needed to trigger signaling pathways involved in conspecific chemical (sexual) communication attributed to this area. Microsc. Res. Tech. 77:313–325, 2014. © 2014 The Authors Microscopy Research and Technique Published by Wiley Periodicals, Inc.

INTRODUCTION Many species that belong to Artiodactyls order show an interdigital pouch or sinus (IS) in all four extremities that can develop into an organ as it occurs in sheep. This organ is considered as a trail gland being important in the production of pheromones (Farillo and Diverio, 2009; Qway and Muller-Scharze, 1970; Sivachelvan et al., 1992). Substances carrying a chemical message among animals are known as pheromones (from the Greek “pherein”—to carry or transfer—and “hormone”—to stimulate or excite) (Karlson and Luscher, 1959). They are active substances secreted to the outside by an individual and received by another one of the same species, in which it evokes a specific behavioral reaction. Pheromone communication plays an important role in mammalian behavior and reproductive signaling acting singly or in combination with sight, olfaction, or tactile stimuli. Pheromones can range from the “releaser” category, which generate behavioral responses (such as sexual attraction and/or copulation), to “primer” pheromones, which generate slower physiological/endocrine or neuroendocrine responses, including hormonal changes that alter reproductive function. In addition, “signaler” pheromones are chemical signals in which information is conveyed but no obvious primer or releaser effect could be established and finally “modulator” pheromones as an additional group of potential chemical signals (Jacob and McClintock, 2000). These chemical substances act as signals and are perceived by the olfactory system and might elicit behavioral and endocrine responses in conspecifics. Many studies have
established the importance of pheromones produced by the male over female reproductive activity, known as the “male effect” (Rekwot et al., 2001). Recent works demonstrate that IS can be considered as an important gland for sexual behavior, as its scent belongs to the list of strong attractive structures to matured males at the height of the breeding season (Takemura et al., 2001). In fact, different body part smells like female urine, external genitalia, modified skin glands such as those present in the ventral tail radix, infraorbital sinus, and IS have strong attractiveness to rams. They induce marked olfactory behaviors in sexually matured males, including the sniffing response to this smell stimulation traduced by head shaking, body movements, and dashing, mainly at estrus (Takemura et al., 2001). We hypothesized that receptors for sexual steroids in IS glands might mediate the glandular secretory response, in different phases of the reproductive cycle. Therefore, the goal of this work was to evaluate morphological aspects of the interdigital pouch of ewes during the estrus cycle at microscopic and ultrastructure levels (SEM and TEM studies), and to determine gene and protein expression of estradiol receptors (ERα and ERβ) and progesterone receptors (P4r), as well as IS biochemical content.

**MATERIALS AND METHODS**

**Sample Collection**

A total of 65 IS were collected post-mortem from adult merino ewes and used for the present work. As the reproductive history of the ewes was unknown, their estrous cycle phases were determined based on ovarian structures and plasma progesterone (P4) concentration. Therefore, when a preovulatory follicle was present in the ovary, in the absence of a corpus luteum (CL), and plasma P4 concentration was below 1 ng/mL, the ewes were considered in the follicular phase. In contrast, when a CL was present and P4 concentration was above 1 ng/mL, the ewe was in the luteal phase. Soon after collection, IS samples were placed in (1) RNAlater (AM7020, Ambion, Applied Biosystems, Carlsbad, CA) for mRNA transcription quantification; (2) 4% buffered formaldehyde, for histology, immunohistochemistry, and confocal microscopy; (3) 4% buffered formaldehyde, for mRNA transcription quantitatively determined using flow cytometry. Receptors location in IS was assessed using laser-scanning confocal microscopy (Leica TCS SP2, Leica Microsystems, Berlin, Germany).

Flow cytometry analysis in SI was performed to quantify the expression of ERs (α and β) and P4r in IS was quantitatively determined using flow cytometry. Receptors location in IS was assessed using laser-scanning confocal microscopy (Leica TCS SP2, Leica Microsystems, Berlin, Germany).

**Histology Techniques**

IS specimens (follicular phase, n = 4; luteal phase, n = 4) were fixed in buffered formaldehyde for 24 h and processed for light microscopic study. Tissue serial sections were cut (5 µm thick—Microm Leica SM2000R) and stained with Weigert Van Gieson for collagen detection, Alcian Blue for mucin detection, and Periodic Acid Schiff to assess glycogen content (Luna, 1992).

**Flow Cytometry Analysis**

Flow cytometry analysis in SI was performed to quantify the expression of ERs and P4rs proteins. SI (estrus n = 12; diestrus n = 12) were removed with a surgical blade and collected into a sterile tube with 1 mL of RPMI 1640 (Gibco-Brl). After disaggregation of the tissue with a surgical blade, samples that corresponded to the total amount of SI mass were centrifuged at 190g for 10 min and resuspended in phosphate-buffered saline solution (PBS). Aliquots of cell suspensions were fixed and permeabilized with FIX & PERM® Fixation and Permeabilization Kit (Invitrogen Laboratories, Life Technologies, Austria) according to the manufacturer’s instructions. Briefly, cells were fixed with Reagent A (Fixation Medium) and incubated for 15 min in the dark at room temperature. After a washing step with PBS and a centrifugation at 274g for 5 min, Reagent B (Permeabilization Medium) was added, as well as the primary antibodies (10 µL), and cells were incubated for 15 min in the dark at room temperature. After a new washing and centrifugation step, RPE-conjugated secondary antibody was added (10 µL) and cells were incubated for 15 min more in the dark at room temperature. A final washing step was necessary.
and after it, the pellet was resuspended in 500 μL of BD FACS Flow (BD Biosciences, San Jose, CA).

Cell acquisition was performed on a BD FACS Calibur flow cytometer (BD Biosciences) and data were analyzed using Paint-A-Gate Pro and Cell-Quest Pro software (BD Biosciences).

In each experiment, cells were also incubated according to the protocol above, but with the secondary antibody only. This control tube was performed in order to access the level of unspecific fluorescence signal of the secondary antibody. All antibodies were previously titrated and the following optimized dilutions and concentrations were used:

Primary antibodies: mouse monoclonal anti-progesterone (77201704 AbD Serotec, UK), diluted at 1:10 in PBS; mouse anti-human monoclonal antibody Er alfa (Ref. 41700, Invitrogen, UK), diluted at 1:10 in PBS; mouse anti-human polyclonal Er Beta2 (MCA2279S, AbD Serotec), diluted at 1:10 in PBS.

Secondary antibody: R-phycocerythrin F(ab')2 frag. of goat anti-mouse (LTI A10543, Invitrogen), diluted at 1:100 in PBS.

**Laser-Scanning Confocal Microscopy**

The same antibodies used in flow cytometry evaluation were used for this study (n = 5). Incubation of antibodies was performed overnight with the following dilutions: (1) Mouse monoclonal anti-progesterone diluted at 1:50; (2) estrogen receptor (ERα) diluted at 1:50 and rabbit anti-human Er diluted at 1:50. FIX & PERM® Fixation and Permeabilization Kit (Invitrogen Laboratories, Life Technologies) was added. Briefly, reagent A (fixation medium) was added for 30 min before the addition of the primary antibody. During another 30 min, solution B (permeabilization medium) was added followed by the addition of the second antibody. To-Pro-3 iodide 1 mM solution (Invitrogen Molecular Probes, Eugene, OR) was used for nuclear counterstaining (n = 5). Negative controls were performed by replacing the primary antibody by either rabbit polyclonal IgG (ab27478, Abcam), for antibodies developed in rabbit, or by mouse IgG (550878, BD Biosciences) for antibodies developed in mouse, in the same dilution and incubation time as the primary antibody, followed by To-Pro-3 iodide for nuclear counterstaining. Selected sections were photographed with confocal laser microscopy, Leica TCS SP2.

**Genomic Assays**

Assessment of P4R, ERα, and ERβ mRNA transcription by conventional PCR in ovine’s interdigital glands (follicular phase, n = 5; luteal phase, n = 5) was possible after specific primer design (Plate 1). RNA was extracted from IS tissue (Qiagen’s Kit for Total RNA Extraction and Purification; ref. 28704, Qiagen, Hilden, Germany) and DNA digested (RNase-free DNase Set; ref. 50979254, Qiagen), according to manufacturer’s instructions. RNA concentration was determined spectrophotometrically (260 and 280 nm) and RNA quality was assessed by visualization of 28S and 18S rRNA bands, after electrophoresis through a 1.5% gel agarose and ethidium bromide staining. Reverse transcription was carried out using Reverse Transcrip-

tase Superscript III enzyme (ref. 18080093, Invitrogen, Gibco, Carlsbad, CA), from 1 μg total RNA in a 20 μL reaction volume, using oligo (dT) primer (27–7858-01, GE Healthcare, Buckinghamshire, UK). Specific primers were chosen for target genes using different Internet-based interfaces, such as Primer-3 (35) and Primer Premier software (Premier Biosoft Int., Palo Alto, CA) (10). Several conventional PCRs were carried out using a default thermocycler (Applied Biosystems) as follows: 2 min at 94°C for denaturation; 35 cycles of 15 s at 94°C for enzyme activation, 45 s at 57–60°C for annealing (depending on the gene—P4R 57, 8°C; ERα 58, 5°C; and ERβ, 60°C) and 45 s at 68°C for extension; and 5 min at 68°C for finalization. To avoid genomic DNA amplification, primers were designed for two different exons and all primers were designed following specific guidelines (Rozen and Skaltsky, 2000, Wang and Seed, 2006). All reactions were carried out in duplicate in 0.2 mL PCR tubes (PCR-0.2-C, Axxygen 321-02-051, CA) in 25 μL reaction volume: 8.5 μL water; 1 μL forward primer (10 pmol/μL); 1 μL reverse primer (10 pmol/μL); 12.5 μL using FideliTaq DNA polymerase master mix (71180, USB, Cleveland, OH); and 2 μL of cDNA. All Agarose (2%) (BIO-41025, Bolin, Luckenwalde, Germany) electrophoresis gel and ethidium bromide (17896, Thermo, Drive Hudson, NH) staining showed a specific and single product.

**Progestosterone Analysis**

Progestosterone concentration in plasma was evaluated using a solid-phase radioimmunoassay (Coat-a-Count Progesterone, Diagnostic Product Corp., Los Angeles, CA). Intra-assay coefficient was 6.4% for the level of 3.2 nmol/L (1 ng/mL) and 4.2% for the level of 15.9 nmol/L (5 ng/mL).

**Fatty Acid Analysis**

Lipids from IS content were extracted from five ewes in the follicular phase according to Folch’s procedure (Folch et al., 1957) but with dichloromethane and methanol (2:1, v/v) instead of chloroform : methanol. The fatty acid methyl esters (FAME) were prepared from the lipid extracts with sodium methoxide in methanol following by hydrochloric acid in methanol (1:1, v/v). The methyl nonadecanoate (19:0) was used as an internal standard. Samples were analyzed using a gas chromatograph HP6890A (Hewlett-Packard, Avondale, PA), equipped with a flame-ionization detector (GLC-FID) and a CP-Sil 88 capillary column (100 m; 0.25 mm i.d.; 0.20 μm film thickness; Agilent Technologies Inc., Santa Clara, CA). The column oven temperature were as follows: initial temperature of 100°C was held for 15 min, increased to 150°C at a rate of 10°C/min and held for 5 min, then increased to 158°C at 1°C/min and held for 30 min, and finally increased to 200°C at a rate of 1°C/min and maintained for 65 min. Helium was used as a carrier gas, and the injector and detector temperatures were 250°C and 280°C, respectively. Identification of FAME was achieved by comparison of the FAME retention times with those of authentic standards (FAME mix 37 components from Supelco Inc., Bellefont, PA). Additional characterization of the FAME was achieved by electron impact (EI) mass...
spectrometry using a Varian Saturn 2200 system (Varian Inc., Walnut Creek, CA) equipped with the same capillary column and with the oven temperatures used for GLC-FID analysis.

**Statistical Analysis**

Weight of IS secretion, as well as flow cytometry data concerning the expression of Ers and P4rs protein in IS from ewes in the follicular and luteal phases, were subjected to a one-way analysis of variance (ANOVA) for unweighed mean values. Significance was defined as values of $P < 0.05$. For statistically different results, the means were further analyzed by post hoc comparison test, such as LSD (least significant differences) and Scheffé (probabilities for post hoc tests).

### RESULTS

The IS is composed of a blind sac and an excretory duct. The IS is located in the space between the digits, surrounded by connective tissue and covered by the interdigital skin, with the duct opening placed on the anterior view of the interdigital space (Fig. 1). When open, the IS shows a surface with thin hair that can be depigmented or dark. The IS amount of secretion was the highest in sheep during the follicular phase compared with the luteal phase ($P < 0.05$) or pregnancy ($P < 0.001$) (Graph 1).

Glandular sebaceous structures occupy a large and superficial area of the pouch. The other glandular department shows a coiled tubular structure (Figs. 2 and 3) and ducts with large diameter (Fig. 4). Secretory cells are tall, columnar with a polyhedral or paved luminal appearance (Figs. 5–7). An irregular luminal surface resulting from the secretory process occurs. Cells showing different stages of differentiation can be observed on which fragments of secretion are being pinched off into the lumen (Figs. 8 and 9), resulting in the formation of collapsed cells. In the same alveolar unit, cells with a different aspect are present at the luminal surface. Some appear quite sharply demarcated by thick rows of microvilli gaining a polygonal aspect (mainly with a hexagonal profile) and some...
secreted material over them. Some others appear rather bulged with apical protrusions with balloon-like swellings that protrude in the glandular lumen and some of these cells appear collapsed by the rupture process associated with the apocrine secretion (Figs. 10–12). Some cells appear to be in an intermediate process between the clear demarcation with surrounding cells by means of rows of microvilli and a progressive filling-inside process that bulges out (Fig. 13a and 13b). Glands generally were depicted as a group of coiled tubules that can exhibit secretory vesicles or secretion blebs and sometimes appeared almost completely filled by a secretory content (Fig. 14). Nevertheless, no changes worth mentioning were detected between IS from ewes in the follicular and luteal phase.

Histology of these glands showed tubule-alveolar units embedded in collagenous tissue. Studies of this sinus according to dye affinities showed that apocrine glands present a mucin content (Fig. 15). These glandular cells are surrounded by a connective sheath (Fig. 16) and a parenchyma rich in glycogen (Fig. 17).

TEM studies pointed out the presence of two different cell types in the apocrine gland (Figs. 18–20). A dark cell with a cytoplasm filled with more electrondense vesicles and another type with electrolucent vacuoles were observed. Vesicles were also depicted in the proximity of myoepithelial cells.

In both follicular and luteal phases, the mRNA for P4R, ERα, and ERβ was shown to be transcribed (Fig. 21). Besides, the presence of both ERs (ERβ and ERα) was demonstrated using confocal microscopy (Figs. 22 and 23–26). Both receptors were present in the cytoplasm of tubuloalveolar cells obtained from the IS of sheep in both follicular and luteal phases. P4R protein was not detected in any phase of the estrous cycle. Nevertheless, all receptor genes were transcribed in IS. Flow cytometric analysis of cell suspensions demonstrated two main populations of cells, with distinct autofluorescence levels and different behavior toward α and β ERs (Fig. 27). One of the IS populations obtained from sheep at the follicular phase expressed higher levels of ERβ compared with ERα (P < 0.05; Graph 2), whereas no difference was observed between them in the luteal phase. When considering ERα and ERβ, they were always more expressed in the follicular than in the luteal phase (P < 0.01; Graph 2).

The fatty acid content was considerably different from the fatty acid composition usually identified in other matrices (as meat and dairy foods). Therefore, it was only possible to identify 42.7% of all fatty acids, due to the absence of equivalent standards. Among the identified portion, 54.7% (wt%) was composed of saturated fatty acids (SFA; C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0, C23:0, C24:0) and 16.4% consisted of monosaturated fatty acids (MUFA; C16:1c9, C18:1c9), whereas the remaining 28.9% were ramified fatty acids (iso and ante-iso fatty acids, i-C12:0, i-C13:0, i-C14:0, i-C15:0, i-C16:0, i-C18:0, and a-C15:0).

**DISCUSSION**

Our work points out the presence of tubule-alveolar units embedded in collagenous tissue inside the IS in accordance to other studies (Karahan et al., 2007).
Glands generally appear as a group of coiled tubules that can exhibit secretory vesicles or secretion blebs. In this study, TEM also demonstrated the presence of two different types of cells in these tubule-alveolar units, as some cells are darker and present a cytoplasm filled with more electrodense vesicles and another type with electrolucent vacuoles. In the same IS, accini have cells with different features, from a sharply demarcated periphery, to a bulged look that can develop into a collapsed appearance, which might be related to secretory function. As known, apocrine secretion entails loss of part of cytoplasm during cell secretion (Charles, 1959), and from a morphological point of view this is revealed by the presence of apical protrusions and/or cytoplasm fragments in the lumen. These protrusions are the result of gradual accumulation of secretory products, which can form balloon-like swellings that protrude into the lumen (Nicander et al., 1974). Independently from the pathway that gives rise to these apical protrusion-forming regions of the plasma membrane, secreted molecules can be synthesized within the cytoplasm and transported to this area giving it a homogenous appearance or apical protrusions that can be filled by many secretory vesicles (Atoji et al., 1993; Wilhelm et al., 1998). In the rat Harderian gland, there are secretory vesicles with similar features to IS, which are released via exocytosis (Brownscheidle and Niewenhuis, 1978; Gesase and Satoh, 2003; Gesase et al., 1996). These findings highlight the occurrence of both exocytosis and apocrine secretory mechanisms in a single cell, although the signaling pathway that triggers vesicular sorting in accordance to the mode of secretion is still unclear (Gesase and Satoh, 2003; Cristofoletti et al., 2001). The presence of a mixed population of epithelial cells in the accini has been demonstrated in the sweat glands of Karagouniko sheep (Pourlis, 2010), exhibiting a paved appearance, while others present different luminal surfaces and simultaneous apocrine and merocrine secretion. In fact, many authors have referred a
Fig. 11 and 12. SEM—The luminal surface of cells can appear quite sharply demarcated by thick rows of microvilli gaining a polygonal aspect (mainly with a hexagonal profile) and some secreted material over them while others appear rather bulged with apical protrusions with balloon-like swellings that protrude in the glandular lumen and some of these cells appear “collapsed” by the rupture process (arrow). Bar = 5 μm.

Fig. 13. SEM—Some cells appear to be in an intermediate process between the clear demarcation with surrounding cells by means of rows of microvilli and a progressive filling-inside process that will rise in the bulge aspect. Cells can exhibit secretory vesicles or secretion blebs. Bar = 5 μm.

Fig. 14. SEM—groups of coiled glands can appear almost completely filled by secretory content. Bar = 50 μm.

Fig. 15. Studies of this sinus according to dyes affinities showed that apocrine glands show a mucin content—Alcian Blue 1000×. [Color figure can be viewed in the online issue, which is available at wileyonelibrary.com.]
puzzling characteristic of most apocrine glands, as they also secrete via exocytosis (Atoji et al., 1988; Groos et al., 1999; Satoh et al., 1992; Wooding, 1980; Zaviacic et al., 2000).

It has been known that in some glands, exocytosis is predominant, whereas in others, apocrine secretion becomes the major pathway for secretion, or in contrast apocrine secretion occurs at a low level compared with exocytosis (Gesase et al., 1996; Payne, 1994). Besides, in some other cases detailed morphological observations are not feasible (Gesase and Satoh, 2003). According to our findings, it seems that in the IS the same acini cells can change from a state of clear demarcation with surrounding cells by means of rows of microvilli, where an exocytosis process of the glandular content release can occur or a mechanism of non-protrusion forming apocrine secretion takes place (Gesase and Satoh, 2003).
It was also interesting to notice the presence of many vesicles in the vicinity of myoepithelial cells, also studied by other authors in apocrine glands (Atoji et al., 1998; Gesase et al., 1995). However, we should take into account that the latter may play a role on supporting glandular end pieces during the secretory process and have lost their importance in the contraction process responsible for apical protrusions formation. In fact, glands that are not associated with myoepithelial cells can secrete via apocrine mechanisms, for example, goblet cells (Kurosumi et al., 1981), whereas other secretory end pieces, which present myoepithelial cells, namely the lacrimal gland, do not secrete via apocrine mechanisms (Satoh et al., 1997).

Even though an increase in the weight of secretions was obtained in the follicular phase, at the ultrastructural level a clear separation among follicular and luteal phase was not seen. Our findings concerning the amount of secretion found inside the IS and its variation according to cyclic phases of the estrous cycle point out its possible role as a scent gland important in sexual communication (Sokolov and Gromov, 1990; Sokolov et al., 1993). Thus, this gland may not only be considered as an important feature implicated in lubrication of the space between the hoof as referred by several authors (Janicki et al., 2003; Jenkinson and Nay, 1975). The amount of this secretion varies, being very sparse or almost absent in pregnant sheep. On the contrary, the secretion was abundant in sheep in the follicular phase and lower at the luteal phase. It is then important to analyze the possible involvement of this structure on the production of scent substances being a specialized organ related to communication not only as a scent trail, which might in fact play a minor role (Hoffman and Thome, 1986), but rather important in sexual communication.

Despite the presence of ERα and PRs in other organs outside the reproductive tract, such as the heart (Arias-Loza et al., 2012; Kararigas-Georgios et al., 2010), liver (Jacob and McClintock, 2000), and intestine (Feng et al., 1993; Tuo et al., 2012), to the best of our knowledge this is the first report on the presence of these receptors in the IS of the ewe. Even though P4R protein was not detected in IS, its receptor genes were transcribed. The fact that in this study mRNA expression of both α and β ERs was increased in the IS at the follicular phase might suggest their involvement on reproduction. As a matter of fact, ERα and ERβ are likely associated olfactory cues in animals (Brown et al., 2010). Administration of specific ERα and ERβ agonists to OVX female mice resulted in their odors mediating male behavior to female cues (Driggers and Segars, 2002). Thus, although ERα has been associated with male sexual behavior, both ERβ and ERα have been linked to sexual and social mechanisms (Hess, 2003; Kavaliers et al., 2012). In addition, in
humans, ERs could influence central and peripheral systems associated with the expression of odor constituents (e.g., vaginal odor constituents—Traish et al., 2010). Therefore, the presence of ER receptors in ewe IS might be also involved in the modulation of odor signals, mainly in the follicular phase, providing information about the condition and identity of the female that can influence males behavior.

One might find unusual the presence of both ERs in the cytoplasm, but in recent years, distinct signaling pathways involving specific complexes of cytoplasmic proteins have been shown to coordinate estrogen action, in alternative to genomic effects of estrogen (attributable to transcriptional activation by ligand receptor), the phosphorylation of the ERs being one of the signals (Driggers and Segars, 2002). In fact, ERs

Fig. 22 and 23. Laser-scanning confocal fluorescence (LSC—lens 63.0× oil) images of the interdigital sinus stained for ERα and labeled with PE. To-Pro-3 iodide was used for nuclear counterstaining. ERα immunoreactivity is clearly observed in cells of the apocrine glands.

Fig. 24 and 25. LSC (63.0× oil) images of the interdigital sinus stained for Erβ and labeled with RPE. In this case, immunoreactivity of the cells is highly detected in cells lining externally the apocrine cells.

Fig. 22. Bar = 18.3 μm and Fig. 23. Bar = 9.9 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 24. Bar = 10.1 μm and Fig. 25. Bar = 10.6 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
were originally regarded to be cytoplasmic receptors in their unliganded form (Gorski and Gannon, 1976) and estrogen signaling has traditionally been identified with the transcriptional control of target genes via the binding of nuclear estrogen receptors to genomic consensus sequences. Moreover, it is also known that there are subtypes of estrogen receptors, each of which may be turned on and off, with different roles on physiological functions and pathogenesis (Wittliff et al., 2013).

Several works have identified biological actions of estrogen that operate too fast to be compatible with transcriptional mechanisms, giving evidence for an important role of non-nuclear estrogen receptor in rapid, nontranscriptional responses of cells to estrogen (Simoncini et al., 2003; Song et al., 2005). These receptors categorized as cytoplasmic, as they act between the cytoplasm and outside of the cell in the cytosol, are responsible for the actions of estrogen, which are faster than the more lengthy processes in which estrogen turns on in the nucleus involving DNA (Cato et al., 2002; Hammes, 2003; Shaaban et al., 2008).

These estrogen receptors might be involved in the process of signaling pathways that might regulate conspecific chemical (sexual) communication attributed to IS as binding of steroid hormone to specific receptors, triggering changes on the rates of nucleic acids and proteins synthesis that might result in the chemosen-sation. These sensory features were developed in order to analyze the chemical properties of the external world, and the detection/discrimination of many kinds of molecules with different chemical structure. The way how hormones influence chemical signaling is in many ways still unclear. Nevertheless, the power of chemosensation has been proved, as demonstrated in the red-sided garter snake (*Thamnophis sirtalis parietalis*), whose males completely rely on the female sex pheromone to identify potential mates among thousands of individuals. In fact, after 1 year of estrogen supplements (17β-estradiol implants), male snakes started secreting a pheromone that seemed to cause other males to swarm to them. These implanted males became attractive and courted by other wild males in outdoor bioassays, and chosen by wild males over those of small females. This behavior was reversed once the implant was removed and indistinguishable from large female trails. Also, estradiol implants increased female pheromone concentration in adult red-sided garter snakes (Parker and Mason, 2012). In another specimen, the Round goby (*Neogobius melanostomus*) odor-ant reactions to estrone were demonstrated, as it elicited male sexual responses at concentrations as low as 4 μg/L (Kolodziej et al., 2003). This might be related to a differential interaction with specific co-activators with specific physiologic functions in different phases of the reproductive cycle.

The fatty acid composition was considerably different from the composition usually identified in other
matrixes (as meat and dairy foods). The complexity of the fatty composition found requires profound study of its composition.

CONCLUSIONS

Although it is consensual that modified skin glands can produce substances that can trigger male olfactory behavior, to the best of our knowledge for the first time it was demonstrated that ERα, ERβ1, and PR are present within the IS. Because the action of estrogens is mediated by specific receptors in target cells, the presence of these receptors are needed to trigger signaling pathways that might be involved in chemical (sexual) communication attributed to this area.

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Declaración of Interest

The authors declare that there is no conflict of interest, which could be perceived as prejudicing the impartiality of the reported research.

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