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Increased squalene concentrations in the clitoral gland during the estrous cycle in rats: an estrus-indicating scent mark?

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Abstract

Squalene in the rat clitoral gland is reported to be semi-volatile and may serve as a chemo-signal. The objective was to determine squalene concentrations in the clitoral gland throughout the reproductive cycle. Clitoral glands were extracted with dichloromethane; 23 compounds were identified with Gas Chromatography linked Mass Spectrometry (GC-MS). Since squalene concentrations were significantly higher during proestrus and estrus, and remarkably reduced during metestrus and diestrus, we inferred that it could be an ovulation-indicating chemosignal in the female rat, acting as a scent mark for the male. This hypothesis was tested by investigating its efficacy to attract males, including studying the role of the olfactory-vomeronasal system of the male in perceiving squalene. For detection of squalene, males used their conventional olfactory system when at a distance from the female, whereas the vomeronasal organ was used when they were in close proximity to the female. We concluded that squalene was a female-specific chemosignal that attracted males, and furthermore, that the olfactory-vomeronasal system had an important role in the perception of squalene.

Keywords: Pheromone; Clitoral gland; Squalene; Estrous cycle; Rat

1. Introduction

The estrous cycle consists of four phases, viz. proestrus, estrus, metestrus, and diestrus. Ovulation occurs during late proestrus to early estrus. The female has to alert males that she is ready for mating [1]; olfactory signals during estrus are used to communicate receptivity [2–4]. Due to their secretive nature, rodents depend solely on chemosignals for sexual attraction. In that regard, sources of chemosignals include urine, faeces, vaginal fluids, and scent gland secretions [5,6]. Regarding the latter, the preputial gland has an important role in scent marking behavior in rodents. This

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gland has been studied in many mammals, including primates, rodents, carnivores, proboscids, and ungulates [7–9].

The preputial gland is formed of modified sebaceous acini and is located on either side of the penis or clitoris in males and females, respectively [10]. In females, this gland is also called the clitoral gland. It is believed that the secretion of this gland is released in two ways: i) via the skin, so it can be smeared on stones, mud, wood, etc., during animal movement, to leave scent marks for counterparts to perceive; and ii) into voided urine. Scent gland secretion is a rich source of lipids involved in communicating information regarding species, sex, dominance status [11–13], as well as for attracting conspecifics [7,14]. It is noteworthy that male rats were more attracted by odors derived from the clitoral gland during estrus than those derived from this gland in other phases of the reproductive cycle [15].

Although scent marks are particularly well-suited for providing reliable signals regarding estrus, it is assumed that their main function is in defense [16]. Recently, Zhang et al [15] reported a high level of squalene in the preputial gland when compared to other glands. In contrast, Natynczuke et al [9] reported the presence of a homologous series of aliphatic acids and their ethyl esters (squalene) in the clitoral gland of brown rats. It is well known that physiological and biochemical profiles of females, particularly lipids, vary according to reproductive status. For example, body odors of female rats contained estrogen-dependent pheromones that attracted males [17,18]. In fact, squalene was reported to be involved in biosynthesis of estrogen during cholesterol breakdown. Therefore, production of squalene in the clitoral gland presumably varied according to phases of the estrous cycle, although this has apparently not been studied.

There are several chemosensory systems that can mediate social recognition in animals through odor detection [19]. In rodents, the main olfactory and vomeronasal system appeared to be of primary importance in this regard [20,21]. It is generally believed that the Main Olfactory System (MOS) was responsible for odor discrimination, whereas the Accessory Olfactory System (AOS) was primarily involved in pheromonal communication [22–25]. The MOS perceives primarily relatively volatile chemical substances (which are air-borne), whereas the AOS deals with non-volatile molecules [26]. Since squalene is semi-volatile, it would be pertinent to determine which of the olfactory systems (i.e., MOS or AOS) perceives squalene if it acts as chemosignal.

To understand chemo-signals in female rats and their importance, it is necessary that the chemicals excreted are collected, analyzed qualitatively and quantitatively, and evaluated for bioactivity. Therefore, the objectives of the present study were to:

i) confirm the presence of squalene in the clitoral gland among phases in the reproductive cycle;
ii) determine the concentration of squalene in the clitoral gland during the estrous cycle;
iii) demonstrate the role of squalene as a chemosignal, adopting a bioactivity-guided assay; and
iv) evaluate the role of the olfactory-Vomeronasal Organ (VNO) in the perception of estrus and non-estrus odors.

2. Materials and methods

2.1. Animals

Twenty adult female Wistar rats, *Rattus norvegicus,* 12–15 wk old, were maintained in polypropylene cages (40 × 25 × 15 cm) with 2 cm of rice husk lining the bottom as bedding material. The rats were housed separately in rooms under laboratory conditions (12 h L:D) without males, and fed pelleted feed (Sai Durga Feeds Ltd., Bangalore, India) and water *ad libitum,* in accordance with guidelines for animal care by the Institutional Animal Ethics Committee (IAEC), Bharathidasan University, Tiruchirappalli, India.

2.2. Determination of the estrous cycle

The estrous cycle was assessed in adult female rats (n = 6), by observing vaginal smears, which were prepared and examined under light microscopy for proportions of the three main cell types, viz. leukocytes, epithelial cells, and cornified cells [27]. At proestrus, the smear has a large proportion of nucleated epithelial cells; at estrus it primarily consists of cornified cells; at metestrus, it consists of an almost equal proportion of leukocytes and cornified cells; and at diestrus, it consists predominantly of leukocytes. This assessment was done, by the same technician, between 07:00 and 08:00 each morning.

2.3. Method of extract preparation

Adult female rats were killed by cervical dislocation under light diethyl ether anesthesia. The clitoral gland was dissected out, placed in dichloromethane, thoroughly homogenized for ~5 min under ice-cold (0 °C) conditions in a sterile glass homogenizer, and centrifuged at 4472 × g for 2 min. The supernatant was immediately filtered through a pre-equilibrated silica

gel column (50 cm). The filtrate was collected in clean glass vials sealed with an airtight, screw-type cap and stored at −20 °C until further analysis.

2.4. Chemical identification by Gas Chromatography and Mass Spectrometry (GC-MS)

The GC-MS analyses were conducted in a QP-5000 (Schimadzu, Kyoto, Japan). An aliquot (2 μL) of extract was injected into the GC-MS on a 30 m glass capillary column, with a film thickness of 0.25 m (30 m × 0.2 mm id, coated with UCON HB 2000) using the following temperature program: initial oven temperature 40 °C for 4 min, increasing to 250 °C at a rate of 15 °C every 10 min. The gas chromatography facility (Schimadzu GC 15A) was equipped with FID detector connected to an integrator. The area under each peak was used for quantification. The detection accuracy was ~1 ng/peak. The relative concentration of each component was recorded as percentage of the ion current. The GC-MS was operated under computer control at 70 eV, using ammonia as reagent gas at 95 ev chemical ionization mode. Identification of unknown compounds was made by probability-based matching, using the computer library within the NICT 12 system.

2.5. Experimental design

2.5.1. Test animals

Eighteen adult male Wistar rats were equally allocated into three groups, intact males (Group I), VNO-ablated (Group II), and $ZnSO_4$-irrigated (Group III).

2.5.2. VNO-ablation and $ZnSO_4$-irrigation

The VNO-ablation was carried out in the males as previously described [28]. Rats were anesthetized under light dose of diethyl ether and 5% $ZnSO_4$ solution was administrated intranasally, to rupture the main olfactory epithelial cells [29].

2.5.3. Odor Preference Test (OPT)

Odor preferences of intact, VNO-ablated and $ZnSO_4$–irrigated male rats were tested. Six sexually experienced male individuals were used for each test, without repetition. The animals were exposed only once to each sample. Fresh samples were used for each trial and the samples were placed in an open Petri dish. The behavioral study was carried out using a "Y" maze apparatus made of tin sheet, and consisting of three arms. Each arm was ~80 cm long and 15 cm wide. The lateral sides were closed with glass plates, whereas the top portion was covered with wire mesh. In this apparatus, feed and water were available *ad libitum*. In the middle of the common space of the apparatus, the responder (i.e., test animal) was released. On the right arm, various samples were kept, i.e., proestrus, estrus, metestrus, diestrus, squalene, and dichloromethane (control). In the left arm, distilled water was kept as control. An odor preference test was conducted; the frequency and duration of visits made by the responders to various samples were recorded separately [30].

Frequency of visit: Number of visits made by the responder towards right or left arm (5 min/test).

Duration of visit: Time spent by the responder in investigation near the Petri dish containing the samples (10 min/test).

Grooming behavior: Number of grooming acts made by the responder near the Petri dish containing the samples (10 min/test).

2.6. Statistical analysis

Data were compiled using SPSS statistical software (Version 10; SPSS Inc., Chicago, IL, USA) and subjected to two-way ANOVA, with post-hoc comparison using Duncan's Multiple Range Test.

3. Results

3.1. GC-MS analysis

The GC-MS profile (Table 1 and Figs. 1–4) were representative compounds obtained from the clitoral gland during the four phases of the estrous cycle. Twenty three peaks were recorded during one cycle. The chemical constituents identified in the samples were alkanes, aldehydes, acids, and amides, with a predominance of alkanes. Visual examination of all chromatograms revealed a consistent qualitative difference in the chemical profiles among the various phases in the estrous cycle; there were only 14 compounds present throughout the estrous cycle (Table 1 and Fig. 1).

The proestrus sample contained 15 peaks and estrus sample contained 14 peaks in which the relative abundance (RA) of squalene was 100. The subsequent metestrus and diestrus rats showed 14 compounds, among which RA of squalene was 24 at metestrus and 48 at diestrus.

Comparison of the compounds, thus identified, across the estrous cycle revealed a remarkable variation. For instance, among the 23 compounds, only one compound, tricosane, was detected only during proestrus and estrus. However, another compound, 9 ehtyl-9-heptyl octadecane, was detected only during the estrus phase. Compounds such as octacosane and octadecanoic acid were present only in the metestrus sample, whereas acetamide

Table 1
List of compounds identified in the clitoral glands of female rats during the estrous cycle.

Peak No.	Retention time	Compound	Chemical class	Pro	Est	Met	Die
1	01.45	Undecane	Alkane	+	−	+	−
2	03.33	2,6-11, trimethyl dodecane	Alkane	+	+	+	+
3	05.78	2 methyl tridecane	Alkane	+	+	+	+
4	08.22	2,6, 11, 15, trimethyl hexa decane	Alkane	+	+	+	+
5	09.70	Trocosane	Alkane	+	+	−	−
6	10.47	Heneicosane	Alkane	+	+	+	+
7	12.52	Pentatricontane	Alkane	+	+	+	+
8	13.15	Pentacosane	Alkane	+	−	−	−
9	14.03	Heptacosane	Alkane	+	−	−	+
10	14.39	Octacosane	Alkane	−	−	+	−
11	14.87	0-nitrobenzaldehyde	Aldehyde	+	−	+	−
12	15.69	Tetratetraconatne	Alkane	+	+	−	+
13	16.10	Demecolcine		−	−	−	+
14	16.48	3-methyl eicosane	Alkane	+	+	+	+
15	17.05	Betulin		−	−	−	+
16	17.25	3 ethyl 5-(2ethylbuthyl octadecane)	Alkane	+	−	+	+
17	17.90	9 ehtyl-9-heptyl octadecane	Alkane	−	+	−	−
18	18.12	Octadecanoic acid	Acid	−	−	+	−
19	18.52,54	Squalene		+	+	+	+
20	19.30	Octadecatrienoic acid	Acid	+	+	+	+
21	20.75	Aristolochic acid	Acid	+	−	−	+
22	23.02	Cholic acid	Acid	+		+	+
23	23.61	Acetamide		−	−	−	+

+, Present; −,Absent; Pro, Proestrus; Est, Estrus; Met, Metestrus; Die, Diestrus.

was found only in the diestrus sample (Table 1 and Figs. 1–4).

Comparison of the RA of compounds also revealed a remarkable difference across the estrous cycle. Interestingly, squalene had high intensity at proestrus and highest intensity during estrus, although it was greatly reduced to below half the intensity at metestrus and diestrus phases (Fig. 3 and Fig. 4). Thus, there was a marked fluctuation in intensity of this compound during the estrous cycle (Figs. 1–4).

3.2. Number of visits

Male rats more frequently visited clitoral gland extract at estrus, followed by proestrus, than metestrus and diestrus (Table 2). A similar trend was noticed in the VNO-ablated rats. Conversely, the $ZnSO_4$-irrigated rats did not show any variation in the preference towards the various samples. Intact, VNO-ablated and

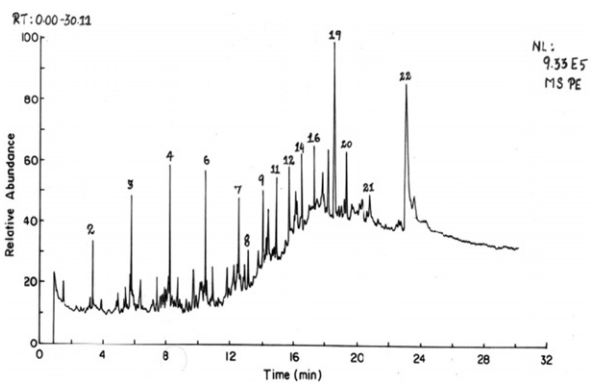

Fig. 1. Gas chromatographic profile of the clitoral glands of proestrus rats. Squalene was expressed at peak 19.

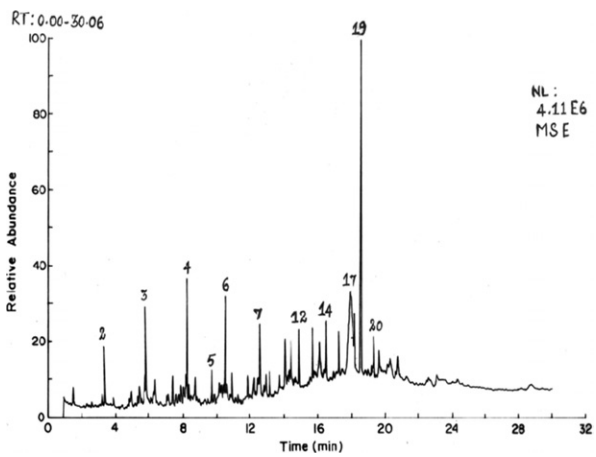

Fig. 2. Gas chromatographic profile of the clitoral glands of estrus rats. Squalene was expressed at peak 19.

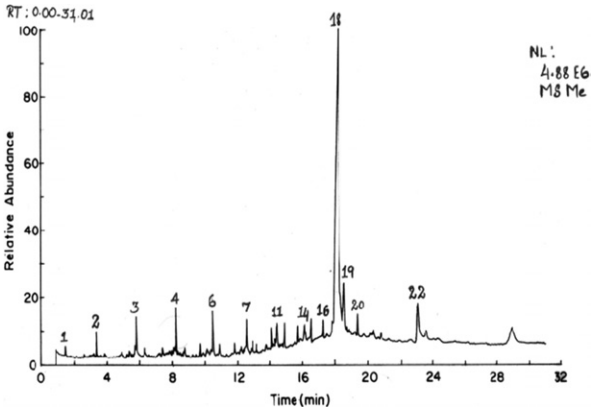

Fig. 3. Gas chromatographic profile of the clitoral glands of metestrus rats. Squalene was expressed at peak 19.

Table 2
Frequency of visits made by three groups of male responder rats towards preputial gland extracts of female rats collected in various phases of the estrous cycle, as well as squalene and dichloromethane (DCM).

Sample	Intact rat	VNO ablated rat	$ZnSO_4$ irrigated rat
Proestrus	5.6 ± 0.2^{b}	4.4 ± 0.6^{b}	1.3 ± 0.5^{b}
Estrus	7.4 ± 1.2^{a}	6.9 ± 0.8^{a}	1.9 ± 0.2^{b}
Metestrus	2.0 ± 0.3^{d}	3.1 ± 0.4^{c}	2.3 ± 1.2^{a}
Diestrus	3.4 ± 0.4^{c}	3.7 ± 0.7^{c}	1.8 ± 0.2^{b}
Squalene	3.3 ± 0.9^{c}	2.0 ± 0.3^{d}	2.6 ± 0.3^{b}
DCM (Control)	0.2 ± 0.0^{e}	0.3 ± 0.2^{e}	0.3 ± 0.1^{c}

Values are expressed as mean ± SEM of six animals, with 5 min/test.
[a-e] Within a column, means without a common superscript differed ($P < 0.05$).

$ZnSO_4$-irrigated rats did not make frequent visits towards squalene, as compared to that of clitoral gland extract. However, none of the male responders exhibited any preference towards dichloromethane. The frequency of visits by the three groups of male responders varied significantly (Table 1). There was a main effect for frequency of visits by all three responders ($P > 0.001$) and the two way interaction was also significant ($F = 29.883$; d.f. 10.54; $P < 0.001$).

3.3. Duration of visits

The duration of visits of rats which visited the sample alone was recorded (Table 3). The time spent by the three sets of responders varied significantly (Tables 2–4). The intact rats spent more time in investigating the proestrus and estrus samples than the other samples. Even though the VNO-ablated rats visited the sample area, the time spent by them was less when compared to the intact rat. In contrast, the $ZnSO_4$- irrigated rats had a trend similar to that of the intact rat, but the response was not equal to that for the estrus samples (Tables 2–4). Interestingly, the intact rats which visited the squalene samples spent more time in investigating the estrus sample; however, only a few rats visited the squalene sample. Although the VNO-ablated rats did not spend much time in the sample area, they spent the most time near squalene. The $ZnSO_4$-irrigated rats showed a similar trend as intact rats. The time spent by all the three responders towards various samples differed with a two way interaction ($P < 0.001$).

3.4. Self-grooming

All responders from all three groups exhibited grooming behavior when exposed to the various samples (Table 4). Intact males spent more time in self-grooming activity during exposure to proestrus and estrus samples than to the other samples. It was noteworthy that intact rats spent more time and exhibited more grooming activity when exposed to squalene than

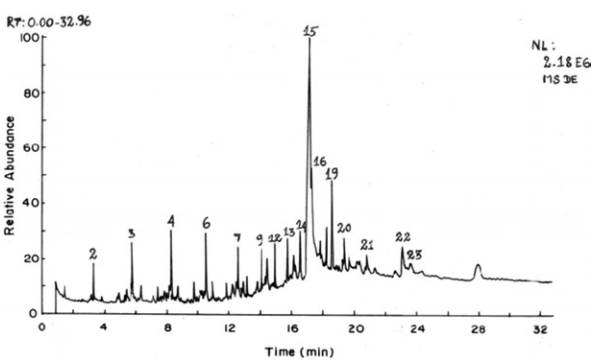

Fig. 4. Gas chromatographic profile of the clitoral glands of diestrus rats. Squalene was expressed at peak 19.

Table 3
Duration of visits (in seconds/10 min/test) made by the responders by the three groups of male responders towards female preputial glands at various stages of the rat cycle, as well as squalene.

Samples	Intact rat	VNO ablated rat	$ZnSO_4$ irrigated rat
Proestrus	428.3 ± 104.3^{b}	128.5 ± 47.2^{c}	289.6 ± 87.4^{c}
Estrus	537.4 ± 142.3^{a}	152.5 ± 62.7^{b}	421.5 ± 134.2^{a}
Metestrus	189.3 ± 83.9^{d}	148.5 ± 105.3^{b}	243.9 ± 64.3^{c}
Diestrus	162.6 ± 68.8^{de}	174.7 ± 63.3^{ab}	126.3 ± 82.4^{e}
Squalene	289.7 ± 89.7^{c}	197.5 ± 127.9^{a}	387.3 ± 105.5^{b}
DCM (Control)	147.5 ± 88.6^{f}	201.3 ± 12.4^{a}	159.3 ± 55.8^{d}

Values are expressed as mean ± SEM of six animals.
[a-f] Within a column, means without a common superscript differed ($P < 0.05$).

Table 4
Grooming behavior exhibited by the responders by the three groups of male responders towards female preputial gland of various stages of rat and squalene (in sec/10 min/test).

Samples	Intact rat	VNO ablated rat	$ZnSO_4$ irrigated rat
Proestrus	45.32 ± 12.75^{c}	18.53 ± 7.29^{c}	67.86 ± 13.46^{b}
Estrus	47.42 ± 7.84^{b}	23.65 ± 10.32^{ab}	59.38 ± 9.53^{c}
Metestrus	29.43 ± 8.61^{e}	18.93 ± 6.67^{c}	$36.49 \pm 14.53_{d}$
Diestrus	37.87 ± 10.62^{d}	19.43 ± 12.65^{c}	28.57 ± 8.04^{e}
Squalene	63.66 ± 4.76^{a}	14.5 ± 5.84^{d}	72.23 ± 6.45^{a}
DCM (control)	18.53 ± 8.45^{f}	25.37 ± 12.83^{a}	29.64 ± 8.94e

Values are expressed as mean ± SEM of six animals.
[a-f] Within a column, means without a common superscript differed ($P < 0.05$).

other samples. The VNO-ablated rats spent more time at the estrus sample than all other samples. However, they spent significantly less time in grooming when compared to intact rats. Similar to intact rats, the $ZnSO_4$-irrigated rats also engaged in grooming activity when exposed to squalene.

4. Discussion

The GC-MS profiles of clitoral gland during the four phases of the female reproductive cycle contained up to 23 volatile compounds. Several of the compounds identified in this study were similar to volatiles identified in urine from the Californian mouse [31] and Swiss mouse [4,32]. The number seemed relatively high compared to the volatiles identified in the preputial gland of house rat, *Rattus rattus,* in which the presence of farnesol was suggested to act as a female attractant [12]. However, farnesol was not detected in the clitoral gland in the present study. In the present study, squalene was detected in the clitoral gland, consistent with a previous report [9]. In contrast, Zhang et al [15] reported the presence of both farnesol and squalene in female and male rats, respectively, and suggested that squalene was a female-attractant produced in excess by the male. It was also suggested that sebaceous and clitoral glands had high levels of squalene, cholesterol esters, and provitamin D (7-dehydrocholestero1) [33]. In the present study, squalene was present during all phases of the estrous cycle, with significantly greater relative abundance during proestrus and estrus, and lesser abundance during metesrus and diestrus.

Estradiol concentrations increase and peak during proestrus and estrus [34]. Fatty acid metabolism is controlled by sex hormones [35]; therefore, it seemed plausible to propose that changes in hormone concentrations may be the basis of variation in squalene production by the clitoral gland during the estrous cycle. Squalene has also been reported to be a major component of the pheromonal scent marks of saddleback tamarins (*Saguinus fuscicollis*), a new world primate [36]. Furthermore, squalene acted as male pheromone in the giant panda, *Aliuropoda melanoleuca* [37].

Interestingly, squalene was present in sweat samples of female humans; its concentration was much lower in premenstrual than menstruating females. Therefore, squalene might be an indicator of ovulation and act as a potential pheromone to attract men [38]. As proestrus and estrus rats are believed to emit pheromonal signals, any compound expressed at a high level specifically during these phases may be a behaviorally important chemical signals that attracts males. Similarly, it was previously reported that urinary volatiles differed only quantitatively, but not qualitatively, during the estrous cycle of mice [39]. However, a specific volatile compound was found in the mouse (1- Iodo 2-methyl undecane) [4] and elephant (7-dodecen 1-yl acetate) [2]. Since squalene is already known to be involved in chemo-signaling, the surge of squalene at ovulation is a candidate for indicating estrus and scent marking. In the present study, squalene concentrations increased during proestrus and peaked during estrus. It is well known that glandular chemo-signals are involved in effective communication [9,10] and, hence, the pheromonal role of the compounds identified in the glandular samples in the present study were confirmed with behavioral analysis.

The male rats made frequent visits towards the vial consisting of clitoral glands of estrous females and spent considerable time near to the sample, suggesting its role in sex attraction. Similarly, male golden hamsters were attracted to the urinary volatiles of females [40]. It is well known that odors of estrus females are usually more attractive to males than those in other phases [40,41]. The attraction to estrus odor by male individuals is presumably due to the presence of specific chemical signals present in estrus female scent sources.

Although the precise functions of the two olfactory systems are not known, there is some evidence indicating they have distinct roles [42]. Although a single system is frequently used for testing, Baxi et al [43] suggested that investigators should use a two-by-two design for testing both the olfactory as well as vomeronasal system to draw a definite conclusion. In the present study, both intact and VNO-ablated rats made frequent visits to the late proestrus and estrus samples, whereas they were not attracted by squalene. This may be due to the semi-volatile nature of this compound. However, $ZnSO_4$-irrigated males were unable to detect

glandular extract as well as squalene, due to the loss of ability to detect volatile odors. In spiny mice, $ZnSO_4$-irrigation eliminated the preference for conspecific's odor, suggesting that the MOS was necessary for individual discrimination [44]. Conversely, the extent of time spent by male rats had different trends. Intact male and $ZnSO_4$-irrigated rats had a preference for the clitoral gland of late proestrus and estrus females, whereas the VNO-ablated rats did not show any significant response. Interestingly, the intact and $ZnSO_4$-irrigated male rats had similar responses towards squalene, in contrast to VNO-ablated rats, which failed to perceive squalene. It is noteworthy that interruption of the olfactory system, particularly the VNO, disrupted reproductive activities in some species of rodents [40,45].

Rodents use self-grooming behavior to encounter their conspecific's scent marks [46]. In the present study, intact males spent considerable time in self-grooming activity during exposure to clitoral gland extracts of late proestrus and estrus phases, as well as to squalene. Interestingly, $ZnSO_4$-irrigated rats spent more time near squalene than near extracts of the estrous female gland. Self-grooming may be another way for the estrus female to convey signals to males. The males would advertise their interest after perceiving female odor by the act of counter-self-grooming [47].

After destruction of the olfactory epithelium, male rats did not make frequent visits towards samples, but the intact rats reached the samples most of the times, except in the case of squalene. Therefore, we concluded that MOS may be involved in perception of volatile signals from a distance, whereas it did not perceive squalene. Thus, squalene may act as chemical signal at close proximity, as perceived by the Vomeronasal System, and not by MOS. Squalene is involved in secondary sexual behaviors such as self-grooming.

It is generally accepted that chemical cues (pheromones) usually contain 5–20 carbon atoms and must be volatile to reach the receiver; the molecular weight of pheromones is usually less than 300 [5]. However, since squalene has a molecular weight $>$ 400 and 30 carbon atoms, it may be only semi-volatile. Since rodents live a secretive mode of life, and the male has to follow the female trail, the persistence of the chemical cues for a prolonged interval is required. In that regard, squalene might persist longer due to its molecular weight. Furthermore, squalene (from the clitoral gland) could be spread by genital grooming, and there could be sufficient heat to promote evaporation of this lipid-derivative. Perhaps squalene may be transferred to the environment, providing markings for males to track females.

In conclusion, squalene, a semi-volatile chemical present in the clitoral gland of the female, reached peak concentrations during estrus, and acted as a chemosignal. In males, the olfactory-vomeronasal system had a synergistic role in the perception of this chemosignal.

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