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Article

Discovery of Boar Pheromones and their Functional Role in Reproduction of Mouse Deer (*Moschiola Indica*)

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Abstract: Two putative boar pheromones (Androstenone and Androstenol) were discovered in endangered mouse deer during captive breeding program. This study further examined the molecular characteristics, pheromone synthesis pathway, and the functional role of these pheromones in reproduction of mouse deer. CYP17A1 and CYB5 genes were cloned and expressed in HEK-293, COS-7 cell lines and gonads of mouse deer to investigate CYP17A1 gene's andien-β-synthase activity towards synthesis of sex pheromones in mouse deer. An enzyme immunoassay was also developed and standardized to measure the fecal androstenone during reproductive cycles of mouse deer. Results showed that mouse deer's CYP17A1 gene possesses andien-β-synthase activity and could transform pregnenolone into 5,16-androstadien-3β-ol. The expression of CYP17A1 gene upregulated in the testis and ovary, compared to other tissues in mouse deer. Significantly elevated pheromones and estrogens were recorded prior to delivery and postpartum estrus / mating in mouse deer. Further, there were weak correlations between fecal pheromones and estrogens/ androgens in mouse deer during breeding season. The findings suggest that the boar pheromones might play a direct role in the reproductive activities of mouse deer which might be used for breeding of mouse deer elsewhere.

Keywords: pheromones; androstenone; mouse deer; metabolic pathway; reproduction; hormones

1. Introduction

Pheromones are chemical messengers secreted by an individual to modulate the behaviour and physiology of conspecifics (1). In mammals, the pheromone plays various important roles in distantly related species such as in social interactions and organization (2), sexual attraction (3), communication between mother and offspring (4), dominance and aggregation behaviour (5), scent marking and territorial behaviour (6). Pheromones are known to influence the secretion of gonadotropin hormones, testosterone, and luteinizing hormones in mice (7), Sea lampreys (8), and sows (9). A significant relationship between pheromones and reproductive hormones in pigs (10) and Asian elephants (11) was observed. Furthermore, pheromones are known to have a direct influence on various behavioural activities during the estrus cycle in gray opossum (12), Asian elephants (13, 11), sows (14), blackbuck (15), mice (16) and bovine (17, 18).

A group of olfactory chemicals known as 16-androstenes were firstly discovered in pigs which play a major role in social, sexual interactions, and reproduction, thus called boar pheromones (19-24). The main component of 16-androstenes (unsaturated C-19 steroids) are 5α-androst-16-en-3-one (Androstenone) and 5α-androst-16-en-3-ol (Androstenol). The 16-androstenes, particularly androstenone, possess a urine-like smell (22) released by the submaxillary salivary gland in large amount and acts as signalling pheromone to stimulate the estrus in female pigs (19, 20, 24). These pheromones are known to have both priming and signalling activity to exhibit potent sexual stimuli in minipig boars

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(25). It is observed that 16-androstenes accelerate puberty in female pigs, known as the "boar effect" (26, 27). The other pheromone, androstenol, produces a musk-like odour and contributes to the biological effects similar to androstenone in sows (14) and humans (28).

Four key candidate genes are involved in the production of 16-androstenes in pigs, including Cytochrome P450C17 (CYP17A1), Cytochrome B5A (CYB5A), 3β hydroxy dehydrogenase (3β-HSD), and 5α reductases (SRD5A) (29). The first step in the biosynthesis of 16-androstenes from pregnenolone is catalysed by the andien-β-synthase enzyme, which in particular converts pregnenolone to 5,16-androstadien-3β-ol (30). The andien-β synthase is the intermediary enzyme between the pregnenolone and 5,16-androstadien-3β-ol, which essentially comprises of CYP17A1, CYB5A, and their associated reductases: NADPH cytochrome P450 reductase (POR) and NADH cytochrome B5 reductase (CYB5R3) (31, 32). The Cytochrome P450c17 possesses two distinct activities; first, the 17α-hydroxylase activity converts pregnenolone to 17α hydroxy pregnenolone and further leads to the synthesis of glucocorticoids, while the second activity, the C17-20 lyase activity breaks the C17-20 bond of C21 steroids by converting 17α-hydroxy pregnenolone to dehydroepiandrosterone (DHEA), the precursor of sex steroids (33).

However, despite these dual activities, CYP17A1 possesses a third specific andien-β-synthase activity that catalyzes the transformation of pregnenolone into 5,16-androstadien-3β-ol, without any intermediate precursors in human and pig (34). CYP17A1 independently shows poor catalysis (< 2%), but it is drastically enhanced by the addition of exogenous CYB5A and POR, revealing 15% increase in pigs and 12% in humans (34). Previous studies have shown that expression of CYP17A1 in pigs is found to be tissue-specific and restricted to gonads (ovary and testis) and adrenal tissues. However, CYP17A1 mRNA expression is equally distributed in the ovary, testis, and adrenal tissues of pigs (35).

Mouse deer (*Moschiola indica*), a primitive deer, belongs to a distinct family, Tragulidae, listed in the Schedule I of the Wildlife protection act 1972, India, is facing severe threats due to habitat loss and hunting. As part of the conservation breeding and species recovery program, the Laboratory for Conservation of Endangered Species, CSIR-CCMB, Hyderabad, is involved with Nehru Zoological Park, Hyderabad, to breed them in captivity. With a founder population of 8 individuals, the breeding centre has bred more than 400 individuals of which more than 100 individuals were released in the wild. We studied the reproductive characteristics of mouse deer as part of the captive breeding program (36) and discovered the occurrence of post-partum estrus within 4-6 hrs of delivery, which is the shortest observed so far among large mammals. This led us to undertake a study on understanding the reproductive physiology of the mouse deer. While analysing hormone profiles using gas chromatography-mass spectrophotometry (GC-MS), we accidently discovered androstenone and androstenol (16-androstenes) putative sex pheromones in endangered mouse deer. With this background, the present study posed the following questions (1) Do these pheromones have a similar molecular pathway of synthesis as reported in pigs and humans? (2) Are these pheromones involved in reproductive activities as previously reported in other mammals? (3) Are these pheromones expressed in specific tissues in males and females? Based on this, the objectives of the present work are (1) To examine the molecular characteristics of sex pheromones in mouse deer (2) To characterize the molecular pathway of sex pheromone production in mouse deer (3) To understand the functional role of sex pheromones and reproductive hormones in the reproduction in mouse deer.

2. Materials and Methods

2.1. Study animal

The experimental group comprised ten adult females and four adult males of mouse deer (n=14) were housed at the mouse deer conservation breeding centre of Nehru Zoological Park, Hyderabad (Table 1). They were housed in 15 (Length) x8 (width) x4 m

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height, covered with semi-natural roofing partially covered with creepers to maintain a natural photoperiod and light. Visibility was maintained between enclosures by iron mesh so that the animals could see other individuals of neighboring cages. Further, these enclosures also had sub-enclosure within it by iron mesh in the corner (3.5 x 4 m) to hold or separate the male or female for some time for collection of samples. These enclosures were enriched with vegetation, including bamboo (*Bambusa vulgaris*), acalypha (*Acalypha indica*), and royal palm (*Roystonea regia*). The animals were fed twice a day with apple (50 g), banana (100 g), carrot (50 g), sweet potato (50 g), soaked grams (50 g), lucerne (100 g), dry grass (500 g) and peepal (500 g) and had free access to clean water as per the Central Zoo Authority of India norms. All behavioral activities, including mating and estrus, were monitored 24X7 using CCTV cameras. Females in an advanced stage of pregnancy were housed with adult males to facilitate mating following parturition (36).

2.2. Fecal sample collection

Fresh fecal pellets were collected in the morning between 9 and 11 am from 14 experimental animals twice a week except for three weeks prior to parturition, when samples were collected daily and stored at -20°C until further analysis. Extreme caution was taken while collecting fecal samples to avoid urine and human sweat contamination. Details of an individual's age, sex, number of samples, reproductive behavior, mating, and other information were recorded (Table 1).

Table 1. Details of animal ID, age, sex, number of fecal pellets collected and date of parturition, mating and postpartum estrus.

S. no	Animal ID/Name	Age (in years)	Sex	No. of samples collected	Date of parturition, and mating observed
1	Reena	5.6	F	161	17-09-2019, 24-02-2020
2	Pinky	3.2	F	179	25-10-2019, 12-04-2020
3	Tejaswini	5.2	F	201	14-12-2019, 19-05-2020
4	Padma	5.4	F	139	24-02-2020, 23-07-2020
5	Vaneja	3.8	F	36	31-05-2020
6	Kavya	1.1	F	130	03-05-2020, 04-11-2020
7	Harshita	3.11	F	87	21-02-2020
8	Sameera	3.1	F	123	12-04-2020, 13-09-2020
9	Rinky	4.1	F	98	-
10	Prameela	5.3	F	123	-
11	Rahul	7	M	85	-
12	Shashank	5.3	M	106	-
13	Vijay	5.2	M	82	-
14	Joshi	3.2	M	77	-

2.3. Tissue collection

Gonad tissues (testes and ovary) and heart tissues were collected opportunistically from Nehru Zoological Park during the study period. The tissues were harvested during post-mortem in RNA*later* stabilization solution (Thermo Fisher Scientific, Waltham, MA, USA) on ice and stored at -80°C until further analysis. For GC-MS analysis, 16-androstenes (androstenone and androstenol) were extracted using the previously modified procedure (37). Approximately 4 g of frozen tissues in triplicates were homogenized using the liquid nitrogen, followed by adding 10 ml of methanol and centrifuging at 4000 rpm for 10 min. The supernatant was transferred into a fresh glass tube, then 3 ml of diethyl ether was added and re-centrifuged to collect the supernatants (Ether layer). The procedure was repeated at least two times. All supernatants were collected in one tube and dried under the stream of nitrogen and resuspended in 20µl of methanol and used in GC-MS.

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2.4. Extraction of androstenone and steroid metabolites

Fecal hormone metabolites were extracted using the previously described procedure (38). The pulverized fine fecal powder was weighed at approximately 0.2 g in a 15 ml glass tube, then 3 ml of 80% methanol was added to it. The samples were vortexed for 30 mins at room temperature and kept overnight at 4°C. The next day, the samples were centrifuged at 3300x *g* for 20 min, transferred to a fresh tube, and kept at -20°C until further analysis.

2.5. Identification of 16-androstenes using GC-MS

The 16-androstenes pheromones were identified using the GC-MS system (Agilent 6900 Agilent technologies, California, USA). Before injecting into GC-MS, approximately 10 ml of pooled fecal extracts were purified by solid-phase extraction using a Sep-Pak C18 matrix column (Waters, Milford, MA, USA). In brief, fecal extracts were passed through the C18 column at 1 ml/5mins flow rate (as per manufacturer's instructions) and eluted with 5 ml of 90% methanol in order to remove the contaminants. The purified supernatant samples were then evaporated under nitrogen gas and resuspended in 100µl of absolute methanol and kept at -20°C until further analysis (39). This method was specifically designed to minimize the loss of volatile 16-androstenes pheromones and to recover the maximum quantity of steroids during purification (37).

Extracted fecal samples were analyzed using a HP-5 MS capillary column with a 30m x 250µm x 0.25µm film thickness (Agilent technologies, California, USA). Helium was used as a carrier gas with a head pressure of 15.8 psi with injector temperature of 300℃. The interface and ion source temperature were maintained at 300 ℃ and 200℃, respectively. The purge valve was turned on 3 min after injection, then 2 µl of each sample extract was injected in the split-less mode with an inlet temperature of 270 °C and after 8 min, the oven temperature was raised to 290 ℃ at 20 ℃ /min and then to 300 ℃ at 15 ℃ for 5 min. The mass spectra were recorded in full scan mode and identified by computer MS library search (Wiley MSD Chem 7th Edition) and authentic standards (Sigma–Aldrich Chemical Co) were used to confirm the desired compounds by comparison of spectra and retention time of samples.

2.6. Total RNA Isolation and cDNA synthesis

Total RNA was extracted from the tissues (testis, ovary, heart) using the trizol (Invitrogen, Carlsbad, CA, USA) method. RNA concentration and quality were evaluated by a ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). After Dnase treatment (Dnase I amplification grade, Invitrogen), cDNA synthesis was carried out using the first-strand cDNA synthesis kit (Takara Bio, Ohtsu, Japan) from 2 µg of the total RNA, and incubated at 42° C for 1 hr, followed by 95° C for 5 min.

2.7. Targetted assembly of candidate gene exons from shotgun sequencing

As no CYP17A1 and CYB5A gene sequences of mouse deer or any closely related species were available in Genbank, shotgun sequencing was performed of mouse deer genomic DNA to assemble the candidate gene exons for designing primers specific to mouse deer. Briefly, genomic DNA was isolated from a tissue sample using DNeasy Blood and Tissue Kit (Qiagen, Germany) and quantified using a dsDNA assay in Qubit 4 Fluorometer (ThermoFisher Scientific, USA). About 1µg of genomic DNA was taken as input for library preparation using Truseq DNA PCR-free kit (Illumina, USA) following the manufacturer's protocol. The purified library was quantified using the NGS library quantification kit (Takara Bio, USA) and pooled with other libraries to obtain about 50x coverage. The pooled libraries were sequenced on an S4 flow cell on the Illumina Novaseq 6000 platform. The first and last exons of CYP17A1 and CYB5A were assembled by taking the corresponding exons from pig reference gene sequences as a template using the selective recursive local assembly of homologous genomic regions (SR Assembler) (40). The primers were designed using the assembled sequences of the first and last exon to amplify

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the whole CDS from the first codon to the last codon, excluding the stop codon. Kozak sequences were added to the forward primer to enable transcription after cloning into an expression vector.

2.8. Construction of CYP17A1 and CYB5A

The cDNA fragments possessing the entire coding region of CYP17A1 and CYB5A of mouse deer and pig testis were amplified using LA taq DNA polymerase (Invitrogen) with primers listed in Table 2. The coding sequences of CYP17A1 and CYB5A with the Kozak regions were then amplified and the segments were cloned into expression vectors pcDNA3.1/V5-His TOPO (Invitrogen) to produce the expression vectors.

Table 2. Primers used for the amplification of sequences of CYP17A1 and CYB5A for PCR, cloning and quantitative PCR for relative quantification of CYP17A1 and β-actin in mouse deer.

S.no	Primer	Forward primer (5′to 3′)	Reverse primer (5′to 3′)	Purpose
1	Mouse deer CYP17A1	ATGTGGGTGCTCGT	CATAGGGTGGAGTTTCGAGG	PCR and Cloning
2	Mouse deer CYB5A	ATGGCCGAGGAG	GTTTTCCGACGTG-TAGAGGTG	PCR and Cloning
3	Pig CYP17A1	ATGTGGGTGCTCTTG	GGAGGTACTCCCCTCAGTG	PCR and Cloning
4	Pig CYB5A	ATGGCCGAACAGT	GTTTTCCGATGTG-TAGAAGTG	PCR and Cloning
5	Mouse deer CYP17A1	TATCATTGACTCCAGCATTGGC	AA-GCGCTCAGGCATGAACAG	qPCR and gene expression
6	β-actin	TCCTTCCTGGGCATGGAATC	GGCGCGATGATCTT-GATCTTC	qPCR and gene expression

2.9. RT-PCR for quantifying CYP17A1 gene expression in tissues

To quantify levels of gene expression of CYP17A1 in different tissues of mouse deer and pig, we designed primers in the conserved exon-exon junctions of mouse deer and pig CYP17A1 and β-actin was used as reference genes for control. The primers spanning the exon-exon junction would amplify only cDNA and will not amplify any contaminated genomic DNA. RT-PCR was performed to examine the expression levels of CYP17A1 testis and ovary in mouse deer using the TB Green Premix Ex Taq II (Takara Bio, Ohtsu, Japan) following the manufacturer's protocol. Heart tissue were used as internal control. The PCR reaction composed of 5μL of SYBR Premix Ex Taq,1 μL of cDNA template, 0.5μL of reverse primer (10μM conc.), 0.5 μL of forward primer (10μM conc.) and 3 μL of milliQ water in a total volume of 10 μL using Roche Lightcycler 480 II (Roche Molecular Diagnostics, Germany). Quantitative PCR conditions were set at 95 °C hot start for 30 sec, followed by 40 cycles at 95 °C for 15 sec and 60 °C for 30 sec, 95 °C for 5 sec and 60 °C for 1 min, and at 50 °C for 30 sec. The PCR amplification products were analyzed by cp value and melting curves.

2.10. Functional characterization of mouse deer CYPA17A1 and CYB5A expressed in mammalian cells

HEK-293 and COS-7 cells were grown in DMEM medium supplemented with 10% fetal bovine serum. Cells were transfected using lipofectamine (Promega) as a transfection agent with pcDNA3.1 (Mock) and pcDNA3.1 encoding mouse deer CYPA17A1 and CYB5A gene construct. After 48 hrs of incubation, fresh medium was added and 50,000 cells/well of 50 μg of ^{3}H pregnenolone was added in the fresh media to the culture plates and kept for incubation at 30°C in 5% Co_2 incubator for 16 hr (41). After incubation, cells were harvested and separated at 1000 x *g* for 5 mins. Further, steroids in the medium were extracted twice with 4ml of diethyl ether, organic phase were pooled and evaporated to dryness using a stream of nitrogen. The extracts were reconstituted with 100 μl of 85% acetonitrile:15% H_2O (41). The separation and identification of steroids were performed using Shimadzu CTO-10AS HPLC-ECD system (Shimadzu Corporation, Tokyo, Japan) on

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a Luna 5µ 100mm x 4.60mm reverse phase C-18 column (Phenomenex, Torrance, CA). The 16-androstenes were separated from pregnenolone using a mobile phase of 85% acetonitrile at 1 mL/min flow rate (41). The presence of 5,16-androstadien-3β-ol in mammalian cells was further confirmed by GC-MS. Respective standards were used as a reference steroid (Steraloids, USA) for the identification of 16-androstenes at 200 nm of wavelength.

2.11. Androstenone antibody production

Androstenone polyclonal antibody was produced to develop the sensitive indirect competitive Enzyme immunoassay (EIA). Two adult New Zealand white rabbits were immunized with 16, (5*α*)-androsten-3-one-carboxymethyloxime: BSA conjugate (Steraloids, USA). The first injection was prepared at a concentration of 1 mg/ml of conjugate dissolved in saline and emulsified with an equal volume of Freund's complete adjuvant (Sigma-Aldrich Chemical Company, St-Louis, MO). The emulsified solution was injected subcutaneously at four sites on the rabbit's back. The booster shots (0.5 mg/ml) were administered with Freund's incomplete adjuvant every two weeks after the initial dose, on days 14, 28, 42, 56, and 70. Blood was collected every one week of booster dose on days 35, 49, 63, and 76. (42, 43). The blood was incubated at room temperature for 4-6 hrs. and vortexed at 4000 rpm to isolate the serum. The polyclonal Immunoglobin (IgG) antibodies were purified using protein-A affinity chromatography (Pierce, India). The final concentration of purified anti-androstenone antibody was found to be 25 mg/ml, divided into aliquots and kept in -80°C. The checkerboard titrations were performed to determine the optimal antibody, conjugated antigen, and secondary antibody dilutions in the androstenone assay.

2.12. Androstenone EIA and procedure

Fecal androstenone was measured using polyclonal anti-androstenone antibody diluted to 1:25600, androstenone standards (5000-10ng/ml), 16(5*α*)-androsten-3-one-carboxymethyloxime: BSA conjugate (1mg/ml) and horseradish peroxidase (HRP)–conjugated goat anti-rabbit IgG secondary antibody (geneilabs, bangalore) diluted to 1:10000. The procedure of 5*α*-androst-16-en-3-one indirect competitive ELISA was performed as previously described (43). The 96 well micro-titer plate (Nunc Maxisorp; Immuno plate, Denmark) was coated with 100 µL of 1µg conjugate/mL of 16 (5*α*)-androsten-3-one-carboxymethyloxime: BSA (diluted in coating buffer, 0.05 M sodium bicarbonate buffer, pH 9.6), placed in a moist chamber and covered with cling wrap and incubated overnight at 4°C. The contents of the plate were discarded and washed four times with wash buffer (0.15M NaCl, 0.05% Tween 20) using an automated ELISA washer (Elx50, BioTek, USA). The plate was blocked with 200µl blocking buffer and incubated at 37°C for 1 hr. The contents of the plate were discarded, blotted the plate and dried in air. Subsequently, 50 µl of the diluted fecal sample (final dilution 1:4 in EIA buffer) and 50 µl of 5*α*-androst-16-en-3-one standards followed by 50 µl of diluted 5*α*-androst-16-en-3-one antibody were added and incubated at 37°C for 90 minutes. The plate was again washed with wash buffer, blotted, and 100µl of HRP conjugated goat anti-rabbit IgG secondary antibody (geneilabs, bangalore) was added to each well and incubated at 37°C for 90 minutes. The plate was washed as mentioned above and 100µl of substrate solution TMB/H2O2 (Tetramethylbenzidine/Hydrogen peroxide, geneilabs) was added and incubated in the dark for 5-10 min (for colour development). The reaction was stopped using 50 µl of stopping solution (1N HCL) and absorbance was read at 450 nm in the ELISA reader (Thermo Multiskan Spectrum Plate Reader, version 2.4.2, Thermo Scientific, Finland).

2.13. EIA for Progesterone, Estradiol and Testosterone

Fecal progestogens, estrogens, and androgens were measured using the previously described methods (44 - 47). Antibodies and HRP conjugates for progesterone (Monoclonal progesterone, Quidel clone no. 425), estradiol (polyclonal estradiol, R0008) and testosterone (polyclonal testosterone, R156/7) were provided by Dr. Coralie Munro (University

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of California, Davis, CA, USA). The monoclonal progesterone antibody was diluted to 1:6000 and 1:100,000 for horseradish peroxidase (HRP) conjugated progesterone and standards (200-0.39 pg/well). Cross-reactivity of progesterone antibody described as previously reported by Graham et al., 2001. The polyclonal estradiol antibody was diluted to 1:10,000 and 1:100,000 for HRP conjugated estradiol and standards (1000–1.95 pg/well). Cross-reactivity of estradiol antibody with estradiol (100%), estrone 3% and < 1% in others (47). The testosterone antibody was diluted to 1:10,000 and 1:2,00,000 for HRP conjugated testosterone and standards (600–1.17 pg/well). Cross-reactivity of testosterone is reported previously (44-46). The procedure for EIAs was performed as previously described (44-46).

2.14. EIA validation for androstenone, progesterone, estradiol and testosterone

The cross-reactivity of 5α-androst-16-en-3-one antibody with other steroids was assessed using the half displacement method as described earlier (48, 43). The androstenone EIA was validated by demonstrating parallel displacement curves between pooled serial dilution of fecal extracts (endogenous) and respective standard (exogenous) to determine the immunological activity of endogenous antigen with the corresponding antibody used in the assay and fecal sample dilution at 50% binding. Antibody sensitivity was calculated for subjective determination at 90% maximum binding. The accuracy or recovery of the assay was measured by adding known amount of unlabelled steroids to the fecal extract. Details of androstenone antibody cross-reactivity to other C19 and various other steroids have been shown in table 2. Antibody sensitivity at 90% binding was found to be 39 ng/ml, 7.8 pg/ml, 23.4 pg/ml and 39 pg/ml for androstenone, progesterone, testosterone and estradiol, respectively

All the EIAs were validated by demonstrating parallelism between pooled standards and serial dilution of fecal extracts (r^2 = 0.99) (Supplementary figure 13). Recovery of known amount of unlabelled steroids were 84.91 ± 4.33, 81.24 ± 2.84, 92.34 ± 5.86 and 98.84 ± 8.51 for androstenone, progesterone, testosterone and estradiol, respectively in fecal extracts analyzed by EIA. The correlation (r^2) and slope (m) value for the exogenous androstenone, progesterone, testosterone and estradiol were r^2 =0.99, m = 0.86; r^2=0.99, m = 0.93; r^2=0.99, m = 1.05; r^2=0.98, m = 1.15, respectively. The intra and inter-assay coefficient of variation (CV) were 6.09% and 10.26% (n=10), 7.64% and 14.63% (n=10), 8.87% and 14.93% (n=10) and 8.385 and 15.89% (n=10) for androstenone, progesterone, testosterone and estradiol, respectively. The presence of androstenone, progesterone, testosterone and estradiol in fecal sample was confirmed by HPLC profile and eluted fractions showed the immunoreactivity of fecal hormones with the corresponding antibody (Supplementary figure 14).

Table 3. Cross-reactivity of Androstenone to other C19 and various steroids.

S. No	Steroid	Cross-reactivity (%)
1.	Androstenone	100.0
2.	4, 16-androstadien-3-one	164.0
3.	Androst-4-ene-3,17-dione	32.7
4.	5α-androst-16-en-3α-ol	12.0
5.	Progesterone	9.44
6.	5, 16-Androstadien-3β-ol	6.47
7.	Testosterone	3.33
8.	5α-dihydrotestosterone	2.0
9.	5-androstenediol	<1.0
10.	Estradiol	<1.0

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2.15. Statistical analysis

Transformation activity assay levels were examined using mean ± SEM of three independent experiments. A post hoc scheffe test was used for comparing transformation levels of CYP17A1 between control vs CYP17A1 in mouse deer and control vs CYP17A1 in pig in HEK-293 and COS-7 cell lines. Hormone values are presented as mean ± SEM. Weekly mean was calculated in all males and females for hormones. Spearman rank correlation coefficient (rs) test was performed to calculate the correlation between individual females androstenone and estrogens and male androstenone and androgens, respectively. Mann–Whitney U test (M–W test) was used for testing differences in fecal progestogens concentrations in pregnant and non-pregnant animals. Statistical analyses were carried out using SPSS 17.0 for hormone analysis.

3. Results

3.1. Identification of 16 androstenes using GC-MS

Following GS-MS mass spectra analysis, two putative 16-androstenes (Androstenone and Androstenol) pheromones were identified in the feces of mouse deer of both sexes (Figure 1 and 2) and also found androstenone in the tissues of testis. The androstenone and androstenol were eluted at 4.97 and 4.78 min, respectively (Figure 1a and 2a). The relative retention times of the feces pheromones were identical with those of androstenone and androstenol standards (Figure 1b, Figure 2b). Androstenone and androstenol showed a base peak value at m/z 79, m/z 257 and m/z79, m/z 148, respectively.

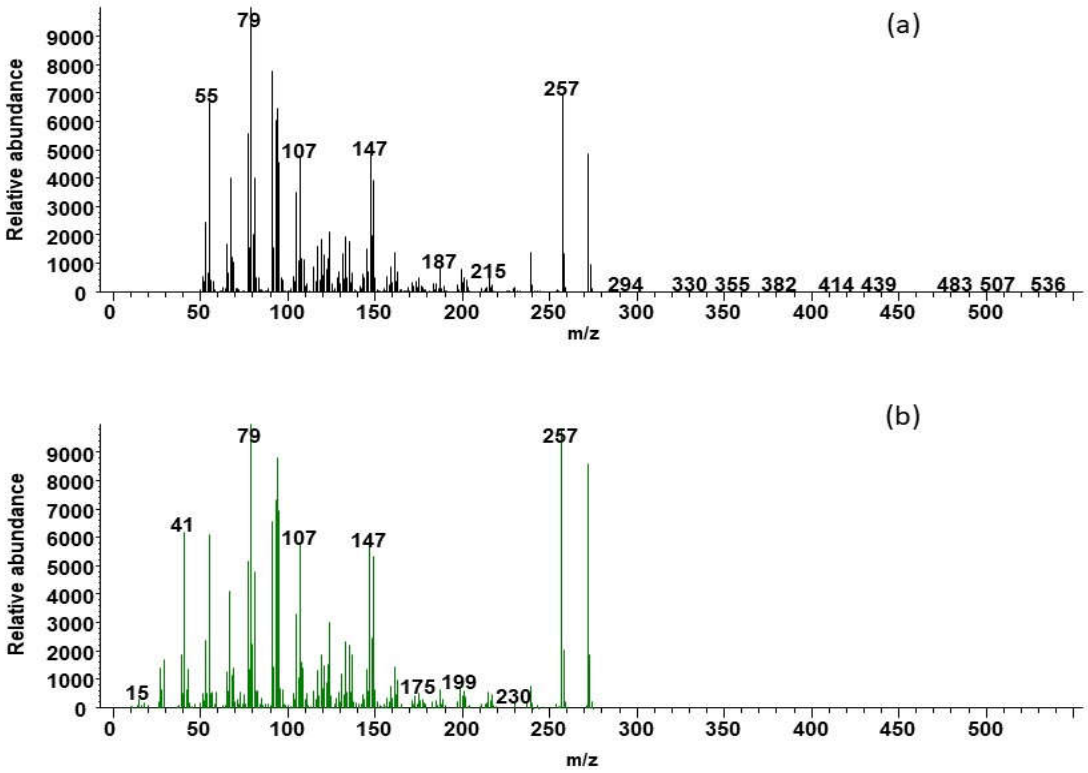

Figure 1. Mass spectra of Androstenone of mouse deer feces (a) compared with synthetic androstenone (b).

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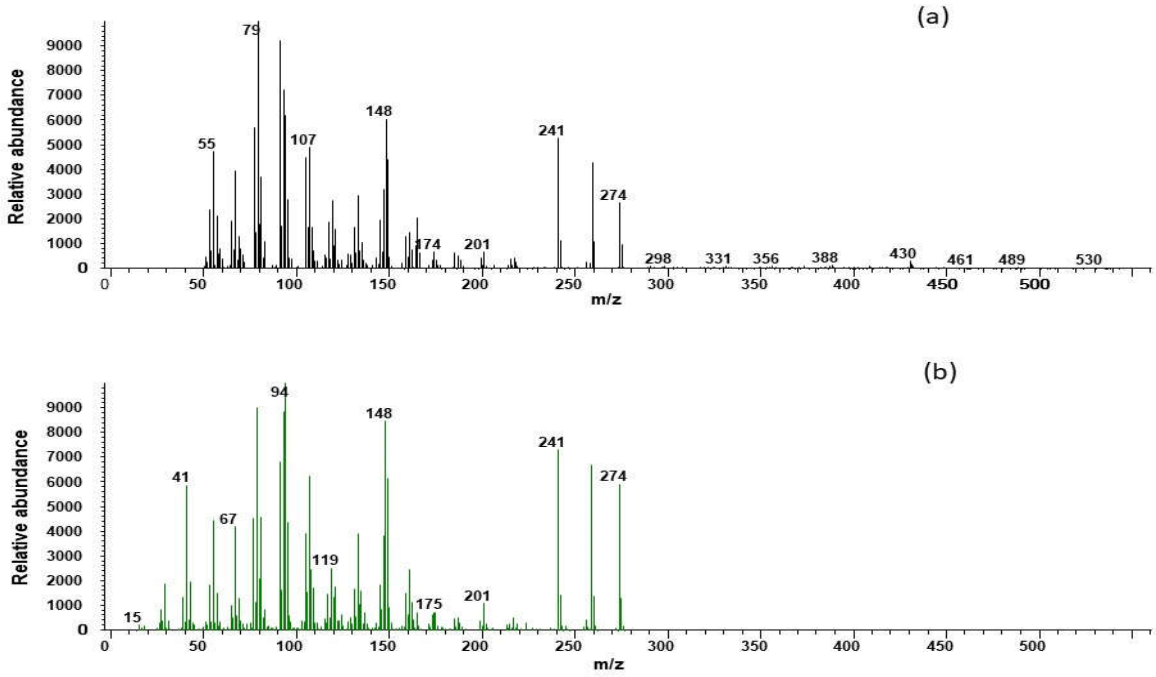

Figure 2. Mass spectra of Androstenol of mouse deer feces (a) compared with synthetic androstenol (b).

3.2. Cloning of CYP17A1 and CYB5A in mouse deer

Fig. 3 shows the deduced amino acid sequence of mouse deer CYP17A1 compared with pig using multiple sequence alignment. The full-length transcript amino acid sequence of mouse deer and pig CYP17A1 were found to be 512 (Genbank accession number: ON804800) and 509 amino acids, respectively. The mouse deer CYP17A1 sequence had 73.8% homology with pig, and it has five extra amino acids (Lys, Leu, His, Pro, Met) as compared to pig. Further there was a deletion of two amino acids at the 21st and 22nd positions of CYP17A1 in mouse deer with reference to pig. In addition, a full-length transcript amino acid sequence of CYB5A in mouse deer was found to be 134 amino acids (Genbank accession number: ON804801) (Fig. 4) which showed 86.57% sequence homology with pig.

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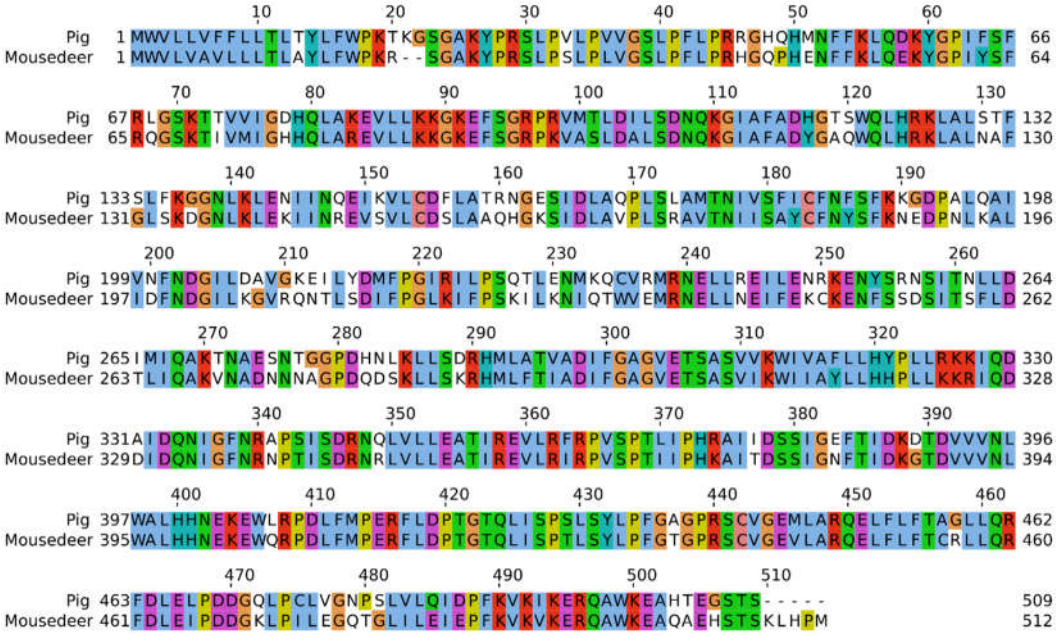

Figure 3. The comparison of deduced amino acid sequence of CYP17A1 between mouse deer and pig. The homologous sequences between mouser deer and pig are highlighted.

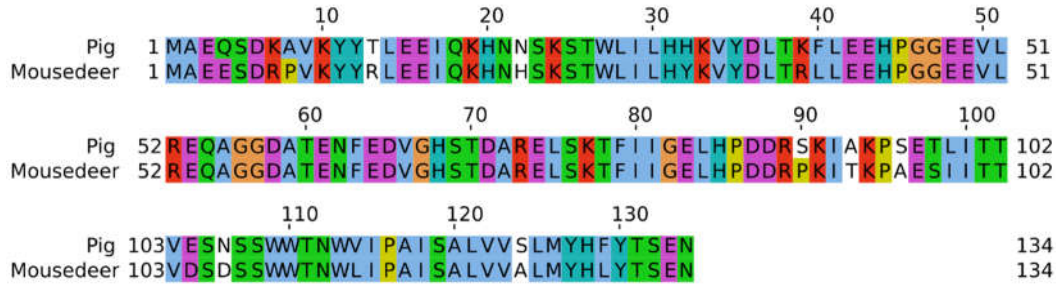

Figure 4. The comparison of deduced amino acid sequence of CYB5 between mouse deer and pig. The homologous sequences between mouser deer and pig are highlighted.

3.3. Conversion of 5,16-androstadien-3β-ol by CYP17A1 mouse deer, in vitro

To evaluate the enzymatic activity of CYP17A1 in mouse deer for conversion of pregnenolone into 5,16-androstadien-3β-ol, two mammalian cell lines; HEK-293, and COS-7 were transfected with expression construct of CYP17A1. In mouse deer, the CYP17A1 enzyme is able to transform pregnenolone into 5,16-androstadien-3β-ol in the presence of cofactors, glucose-6-phosphate salt, glucose-6-phosphate dehydrogenase, and nicotinamide adenine dinucleotide phosphate (NADP) salt (Fig. 5). However, the observed transformation was less than 1%. The transformation of 5,16-androstadien-3β-ol from pregnenolone was significantly higher in mouse deer and pig than control in HEK-293 cell-line (Post hoc scheffe test; P = 0.024, n=5) and COS-7 cell-line (Post hoc scheffe test; P = 0.039, n=5). Interestingly, no increase was found in 5,16-androstadien-3β-ol in the presence of CYB5A. Further, no significant difference was observed in the transformation of 5,16-androstadien-3β-ol in HEK-293 and COS-7 cells (M-W U=14; P=0.9). Overall, results showed that mouse deer P450C17 has a catalytic activity to transform 5,16-androstadien-3β-ol in the absence of oxidoreductase enzymes POR and CYB5R.

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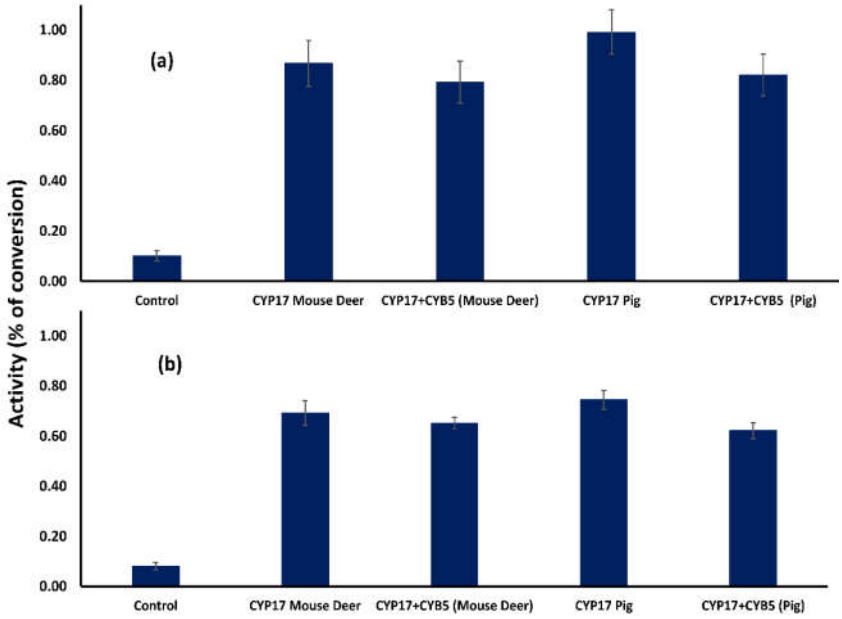

Figure 5. Transformation of 5,16-androstadien-3β-ol from pregnenolone by mouse deer and pig CYP17A1 and effect of CYB5A in combination with CYP17A1 in (a) HEK-293 and (b) COS-7 cells. The results are the mean ± SEM of three independent experiments. An empty vector was used as a control.

3.4. Relative mRNA transcript expression of CYP17A1 in testis and ovary of mouse deer

Mouse deer CYP17A1 mRNA transcript shows tissue-specific expression in testis and ovary as compared to heart (as a control) Figure 6. The expression of CYP17A1 mRNA was restricted to gonads of mouse deer and did not express in the heart tissue of mouse deer.

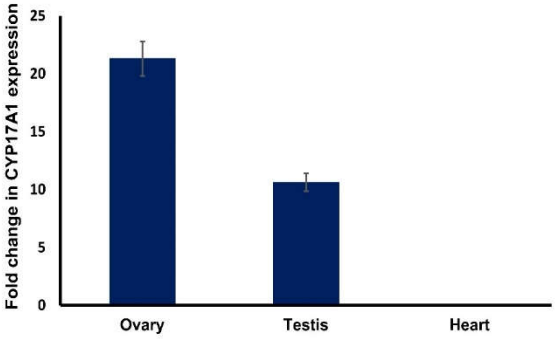

Figure 6. Normalised expression of mouse deer CYP17A1 with respect to β-actin in ovary, testis and heart**.**

3.5. Reproductive monitoring

A total of 1627 fecal pellets were collected from 10 adult females and four adult males mouse deer for 12 months (Table1). Of the 10 females, eight females were observed with 13 parturitions, post-partum estrus observations, and successful mating (Table1) during the one year of the study period.

3.6. Fecal androstenone profile during parturition, postpartum estrus, and mating

The mean fecal androstenone concentrations ranged between 47.66 ± 4.08 ng/g and 226.11 ± 14.55 ng/g. Fig. 7 shows the fecal androstenone concentrations (weekly mean +Standard error of mean (SEM)) of adult female mouse deer from 11 weeks prior to parturition to 11 weeks post parturition, 0 week indicates the week of parturition and mating.

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A gradual increase of fecal androstenone concentrations began on the third week and led to a sharp increase on day 0 (equivalent of week -3 to day 0 in Fig. 7) (third week vs. zero-week, M-W U = 34, P = 0.03, n=8; second week vs. zero-week, M-W U = 43, P = 0.05, n=8) and significantly decreased on the third week from 0 week (zero-week vs third week, M-W U=30, P = 0.03, n=8). In addition, the presence of androstenone was confirmed in fecal sample and examined androstenone antibody immunoreactivity against the fecal androstenone using High Performance liquid chromatography (HPLC).

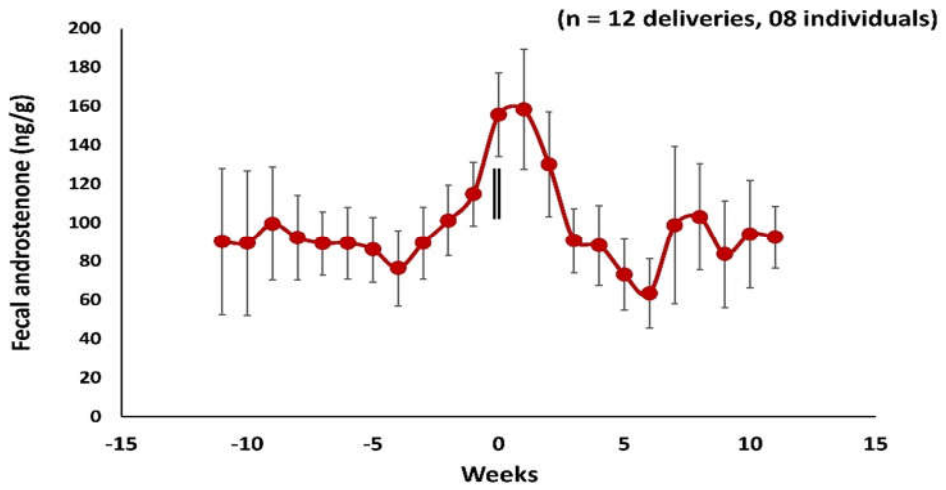

Figure 7. Profile of mean ± SEM weekly fecal androstenone in mouse deer during 11 weeks before and post parturition. The 0 week and vertical bars indicate the parturition, mating, and postpartum estrus (n=12) were recorded in eight individuals.

3.7. Fecal estrogens during postpartum estrus and mating

Overall, fecal estradiol metabolite concentration ranged widely from 57.10 ± 2.65 ng/g to 189.91 ± 8.42 ng/g (Fig. 8). There was a transient rise of fecal estrogens in females exhibiting estrus. Fecal estrogens levels were elevated for about 3 weeks prior parturition, peaked at the day of postpartum estrus and then declining by 3 weeks of parturition (-3 vs -1-week, M-W U = 31, P= 0.05, n=8; -3 vs 0-week, M-W U = 30, P=0.04, n=8; 0-week vs 3-week, M-W U = 25, P =0.03, n=8). However, all individual females show estrogens peak within 1.33 ± 0.18 (n=12) days following the delivery. The peak observed in fecal estrogens levels could be of follicular origin as mating was observed within 4-6 hrs of parturition.

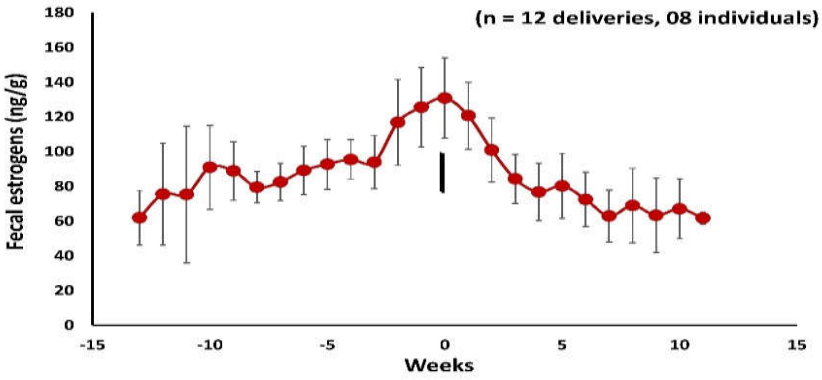

Figure 8. Profile of mean ± SEM weekly fecal estrogens in mouse deer during 11 weeks before and after parturition. The 0 week and vertical bars indicate the parturition, mating, and postpartum estrus (n=12) were recorded in eight individuals.

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3.8. Fecal androstenone vs. fecal estrogens

Overall, 10 out of 13 deliveries from 6 female individuals showed a weak positive correlation between fecal androstenone and estrogens concentrations one week prior to parturition, postpartum estrus and mating (rs=0.78, P=0.008; rs=0.24, P=0.489; rs=0.87, P=0.001; rs=0.24, P=0.489; rs=0.18, P=0.603; rs=0.40, P=0.244; rs=0.09, P=0.803; rs=0.30, P=0.385; rs=0.15, P=0.676; rs=0.22, P=0.533, n=6 individuals, 10 deliveries, Figure 9)

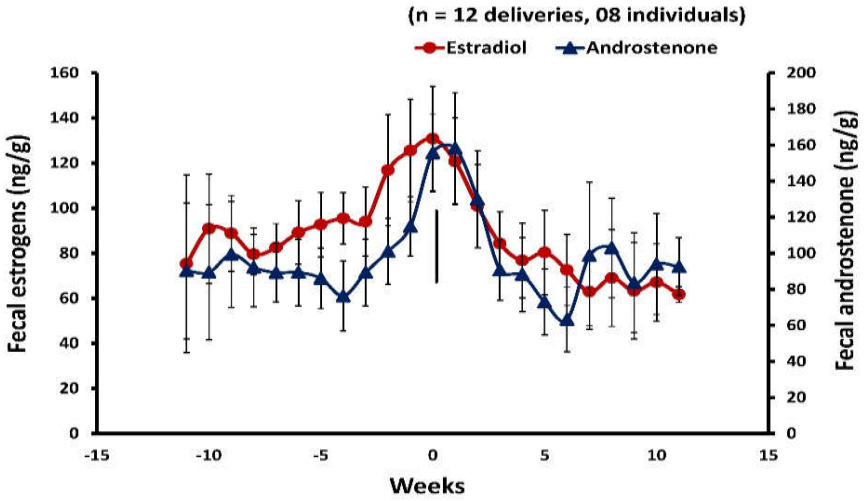

Figure 9. Profile of mean ± SEM weekly fecal androstenone vs estrogens in mouse deer during 11 weeks before and after parturition. The 0 week and vertical bars indicate the parturition, mating, and postpartum estrus (n=12) were recorded in eight individuals.

3.9. Fecal androstenone and testosterone in males

Individual mean fecal androstenone concentrations ranged from 5560.84 ± 278.44 ng/g to 16027.87 ± 1109.119 ng/g and began increasing 5-6 weeks prior to mating. Moreover, individual mean fecal androgens concentrations ranged from 311.31 ± 29.85 ng/g to 1069.76 ± 26.32 ng/g (Figure 10). Overall, all individual males showed a weak positive correlation between fecal androstenone and androgens concentrations (rs=0.90, P=0.002; rs=0.42, P=0.289; rs=0.476, P=0.233; rs=0.762, P=0.028; rs=0.262, P=0.531; rs=0.929, P=0.001, n=3 individuals, 6 deliveries)

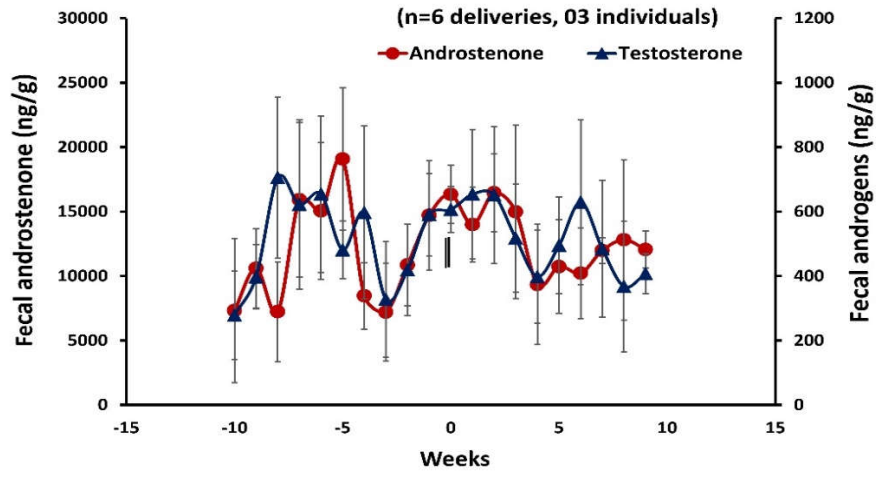

Figure 10. Profile of mean ± SEM weekly fecal androstenone and androgens in male mouse deer during 10 weeks with respect to parturition in females. The 0 week and vertical bars indicate the parturition and mating in females.

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3.10. Fecal progestogens profile during pregnancy and post-partum estrus

Overall, fecal progesterone metabolite concentrations ranged from 4116 ± 204.97 to 20175 ± 1083.92 ng/g (Figure 11). The mean fecal progesterone metabolite concentrations declined significantly from the day of parturition from 18067 to 10207 ng/g (-1 week vs. 1 week, M-W U = 22, P=0.00, n=8, Figure 10.).

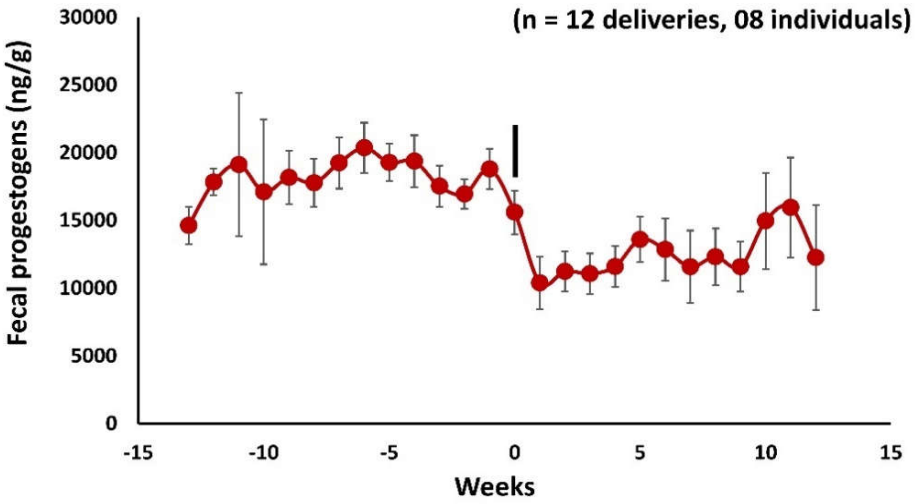

Figure 11. Profile of mean ± SEM weekly fecal progestogens in mouse deer during 11 weeks before and after parturition. The 0 week and vertical bars indicate the parturition, mating, and postpartum estrus (n=12) were recorded in eight individuals.

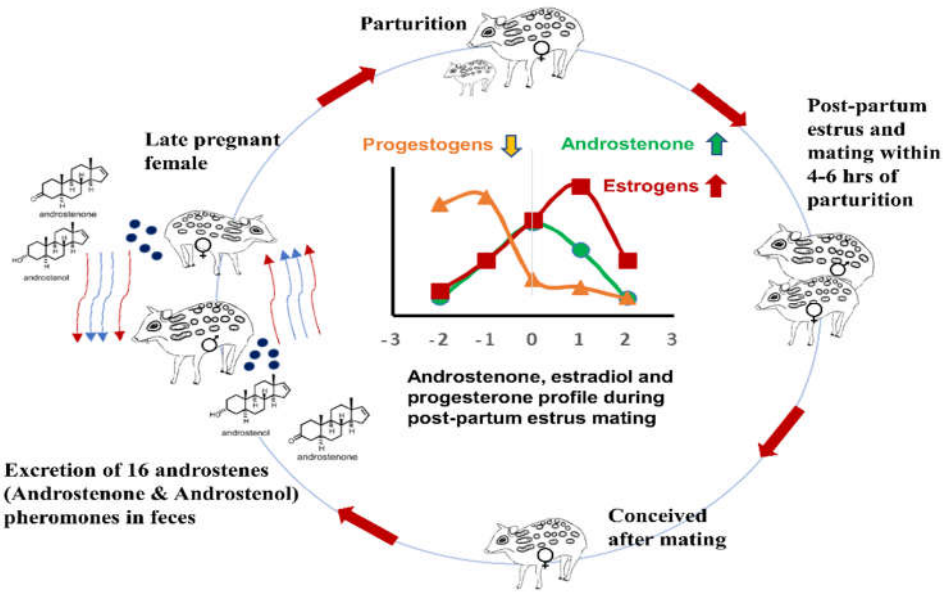

Figure 12. The graphical representation of a model how androstenone (16 androstenes), estrogens and progestogens play a crucial role during parturition, post-partum estrus and mating in mouse deer.

4. Discussion

Pheromones play major role in reproduction in mammals (13, 7, 9), however, a few studies have demonstrated the pheromone synthesis molecular pathways and their specified role in reproduction. In this study, two putative sex pheromones (androstenone and androstenol) which we identified in mouse deer showed that mouse deer P450C17

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gene possess an andien-β-synthase activity to transform pregnenolone to 5,16-androstadien-3β-ol, Although he 5,16-androstadien-3β-ol is considered to be an intermediate and precursor in the 16 androstene pathway leading to the biosynthesis of androstenone and androstenol (34). For the first time, we show that POR and CYB5R are not essential for the conversion of pregnenolone to 5,16-androstadien-3β-ol using P450C17 in mouse deer and pigs. Soucy et al., 2003 (34) showed that pigs and humans CYP17A1 possess andien-β-synthase activity that catalyzes the conversion of 5,16-androstadien-3β-ol from pregnenolone. However, the conversion of 5,16-androstadien-3β-ol from pregnenolone was poorly catalysed (<2%) in the presence of POR. Furthermore, CYB5A stimulates and enhance the formation of androstadienol in combination with P450c17 and POR in human (12%) and pig (15%).

It has been previously shown that CYP17A1 catalyzes the production of 17-α-hydroxyprogesterone (17αOHP) and DHEA from pregnenolone using the 17α-hydroxylase and C17,20 lyase reactions, including the conservation of 5,16-androstadien-3β-ol in a single step via andien-β-synthase pathway (49, 31, 32). In addition, CYB5A and CYB5A reductase can modulate P450C17 enzyme activities as CYB5A allosterically interacts with the CYP17A1-POR complex to stimulate the production of 5,16-androstadien-3β-ol (50). In this study, CYB5A in combination with P450C17 does not stimulate the synthesis of 5,16-androstadien-3β-ol from pregnenolone when POR and CYB5 reductase were omitted from the transfection assay. Moreover, we could not detect DHEA conversion from pregnenolone in HEK-293 and COS-7 cells with and without CYB5A using CYP17A1. It might be due to the lack of CYP17A1-POR complex for receiving and transferring the electrons as POR transfers electrons from NADP to microsomal P450 enzymes and only redox partner for all microsomal P450s enzymes (51). Moreover, CYB5A accepts an electron from POR or CYB5A reductase and transfers them to CYP17A1 (52). It is also noted that CYB5A neither stimulate nor inhibit the catalytic activity of CYP17A1 depending on the substrate and specific enzymes involved in the reaction (53). Overall, our results showed that mouse deer CYP17A1 catalyzes the formation of 5,16-androstadien-3β-ol from pregnenolone using glucose-6-phosphate salt, glucose-6-phosphate dehydrogenase, and NADP salt cofactors

The homology of the CYP17A1 amino acid sequences between mouse deer and pig was 73.87%, with five extra amino acids and deletion of two amino acids at the 21st and 22nd positions in mouse deer as compared to pig. The mouse deer's CYP17A1 was able to transform pregnenolone to 5,16-androstadien-3β-ol with observed additions and deletions of amino acids. We have also observed that the CYP17A1 in humans (508aa) and pigs (509aa) have a single amino acid difference but such difference would not affect the enzymatic activity for the conversion of 5,16-androstadien-3β-ol from pregnenolone (54, 55). Furthermore, to investigate the effects of additions and deletions of amino acids in mouse deer CYP17A1, we performed expression of mouse deer CYP17A1 and found that expression was upregulated and restricted to gonads (testis and ovary) compared to other tissues (heart). However, the expression was higher in the ovary than testis. Robic et al [36] showed that mRNA expression of pig CYP17A1 was restricted to testis, ovary, and adrenal tissues and equally distributed among them

To support the hypothesis that androstenone plays a pivotal role in the reproduction of mouse deer, an enzyme immunoassay was specifically developed and standardized by raising the antibody against androstenone to measure the fecal androstenone using a non-invasive method. The fecal androstenone concentration followed a cyclic pattern in which delivery and postpartum estrus could be clearly distinguished from consistently elevated androstenone levels compared to baseline prior to parturition, postpartum estrus and mating. Dehnhard et al., 2001 (11) identified two volatile androgen-based pheromones 5a-androst-2-en-17-one and 5a-androst-2-en-17b-ol, in the urine of female Asian elephants, which induced behavioural responses in elephant bulls. It has been demonstrated that these two urinary pheromones were involved in the estrus-related activity and positively associated with urinary pregnanetriol to indicate the luteal phase and reflected the ovarian cyclicity in females ((11)). Based on the significant higher concentrations of fecal

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androstenone during parturition and mating, it can be inferred that androstenone plays a major role in postpartum estrus and mating behaviour in female mouse deer either alone or in coordination with estrogens. Moreover, all individual females showed estrogens peak within 1.33 ± 0.18 (n=12) days following parturition. However, the peak in fecal estrogens levels could be of follicular origin and presumably associated with luteinizing hormones as mating was observed within 4-6 hrs of parturition. A similar observation was reported in tammar wallabies, whereby mating occurred within the first 1-6 hr (1.3 ± 0.8 hrs) of birth (56). In addition, serum estradiol measured in tammar wallabies showed a rise in estradiol levels 2 days prior to parturition and peaked 1-2 days postpartum and declined by day 3 post-partum. The peak in serum estradiol levels was suggested to be of follicular origin (57). In tammar wallabies, ovulation usually occurs between 43 and 60 hrs of postpartum (58) and sperm reach the cervix within 40 mins of copulation, the uterus by 4 hrs, lower oviduct in 6 hrs and maintained in the uterus for over 40 hrs (59-61). Similar scenario could be hypothesized for mouse deer that after successful copulation, spermatozoa are presumably stored in few hours in the reproductive tract until ovulation and fertilization occur. The phenomenon of storage of sperm in a specialized structure of the reproductive tract in females are well known and reported in many species (62).

In addition, the present study observed a positive correlation in 10 deliveries out of 13 (n=6) between mean fecal androstenone and estrogens concentrations one week prior to parturition and postpartum estrus. This positive association could be due to the relationship of the combined synthesis of closely related sex steroids and pheromones. Babol et al., 1999(10) reported a positive correlation between plasma androstenone and the synthesis of plasma androgens and estrogens in male pigs. Estrogens are produced from androgens by aromatization using aromatase enzyme and are involved in the regulation of accessory sex glands of the male pig (63). In addition, salivary gland and fat showed a positive correlation between androstenone, androgens, and estrogens in pigs (64, 65). Similarly, adult males which were caged together with females for mating during parturition, showed a positive correlation in all individuals (n=3) between fecal androstenone and androgens one week prior to mating in females. As fecal estrogens and androstenone peak precisely followed the estrus and the decline in progesterone levels is an effective stimulant for estrus behaviour and provides necessary preconditions for estrus and mating. Our hormone analysis also supports the previous findings on postpartum estrus in female mouse deer (36).

In conclusion, the current study sheds new light on how 16 androstenes plays a major role in reproduction of mouse deer by demonstrating a molecular pathway of pheromones synthesis and long-term monitoring of androstenone using non-invasive method in captive populations. Based on finding, we hypothesis that 16 androstenes have a major role in post-partum oestrus and mating as mouse deer are solitary animals and presumably use 16-androstene pheromones for sexual communication and advertising their reproductive status to the opposite-sex in wild conditions.

Supplementary Materials: All the enzyme immunoassays were validated by demonstrating parallelism between pooled standards and serial dilution of fecal extracts ($r^2 = 0.99$) (Supplementary figure 13).

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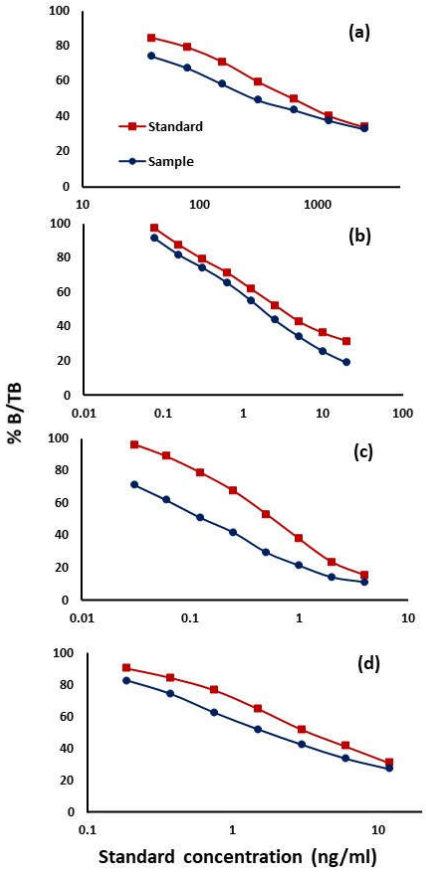

Figure S13. Parallel displacement curves between the pooled serial dilution of fecal extracts of mouse deer (square) and respective standards (circle) of (a) Androstenone (b) Estrogens (c) Progestogens (d) Androgens.

High-performance liquid chromatography for EIAs validation

HPLC was performed to identification, separation, and evaluation of immunoreactivity of fecal androstenone, progesterone, estradiol, and testosterone with the corresponding antibodies using the Shimadzu CTO-10AS system (Shimadzu Corporation, Tokyo, Japan). Fecal hormones were separated and identified using steroid-specific reverse-phase C-18 column (waters column, symmetry C-18, 4.6 x 20 mm, 3.5 mm, intelligent speed (IS) column. Prior to HPLC, pooled fecal samples were purified and passed through Sep-Pak C18 cartridges (Waters, Milford, MA, USA) and eluted with absolute methanol as described previously (45). The purified supernatant was dried in nitrogen gas, reconstituted in 100µl of absolute methanol and vortexed for 1 min. Respective standards and fecal samples were injected into the HPLC and eluted using a gradient flow of 20–94% acetonitrile (ACN): water (H2O) for 8 min at a flow rate of 1 mL/min. Hormones were detected at the 190 to 400 nm wavelength and eluted fractions of 250µl were collected every 15 seconds (4 fractions/minute) and vacuum dried. The dried samples were resuspended in 100 µL of EIA buffer to evaluate the immunoreactivity using the corresponding hormone EIA (Figure S14).

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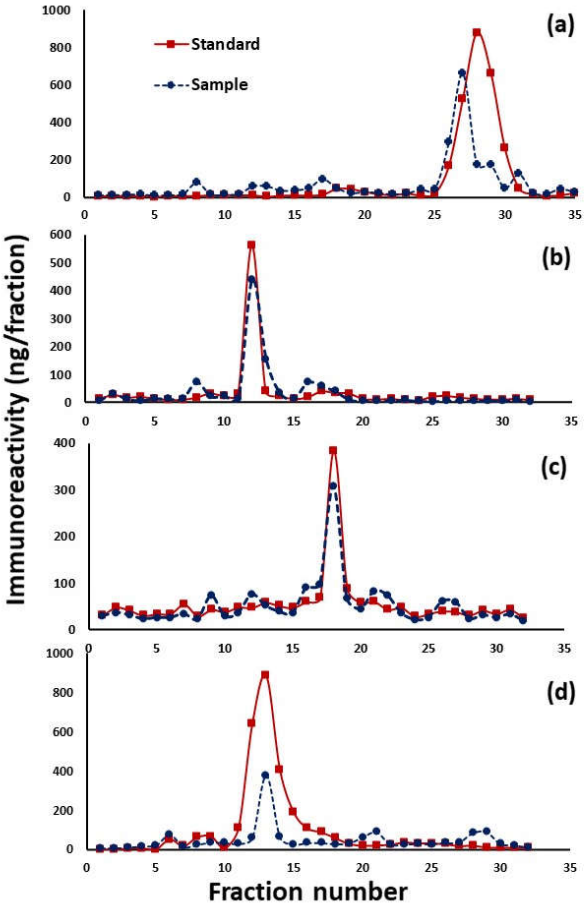

Figure S14. High-performance liquid chromatography separation of immunoreactive (a) Androstenone (b) Estrogens (c) Progestogens (d) Androgens in fecal extracts (dark line) of mouse deer and respective standards (dotted line).

Author Contributions: GU, BS and VK conceived, designed, and supervised the study. GU and BS contributed reagents and materials. VK, SM, KC, AS, SKM, MS, WD analyzed and interpreted the data. VK wrote the manuscript with input from SM, SKM, WD, GU and BS. All authors critically read and approved the final manuscript.

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Data Availability Statement: The data analyzed during the current study are available from the corresponding author on reasonable request. Further, the sequence datasets generated and analysed during the current study are also available in the GenBank repository, https://www.ncbi.nlm.nih.gov/genbank, with accession numbers provided in this article.

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Conflicts of Interest: The authors declare no conflicts of interests.

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