Chapter I

DETECTION OF LOW MOLECULAR MASS PROTEINS AND VOLATILE COMPOUNDS IN THE PREPUTIAL GLAND AND THEIR ENDOCRINE DEPENDENCY

1.1. INTRODUCTION

Nocturnal habits and dark living environments have led to the evolution of olfaction as a major tool for animal communication in rodents (Robertson et al., 1993). Olfactory communication is essential to find energy and primary metabolites, avoid toxic substances and withdraw hostile environments. As regards animals, it is also vital for inter-individual communication among the members of same species, which favours reproduction and social life organization. All mammals emit chemical cues in their environment via urine, faeces and secretions from scent glands (Brennan and Keverne, 2004; van den Hurk, 2007).

A plethora of chemical substances is synthesized in mammalian scent glands that, in turn, are distributed in a wide range of anatomical sites. In general, however, those secretory glands associated with the genitalia elaborate odorants related to reproductive behaviour, while those located on ventral, dorsal, head and flank regions secrete compounds related to territorial marking and other behaviours associated with social status (Stoddart, 1980; Pietras, 1981). Among the scent glands, the well known odorant producing organs in mammals is the preputial gland, found in many non-primate vertebrates (Adams, 1980). For many species including several ungulates (Odend’hal et al., 1996), musk ox (Flood et al., 1989), white tailed deer (Gosling, 1985), musk deer (Sokolov et al., 1987) and rodent species (Kannan et al., 1998; Kannan and Archunan, 1999, 2001; Zhang et al., 2007a,b; 2008; Pohorecky et al., 2008), but not primates (Comfort, 1974; Keverne, 1980), the preputial gland is most important for reproductive pheromone/scent production. Further, it is reported that the preputial glands have been shown to be a source of primer pheromonal effect...
(Marchlewksa-Koj et al., 1990; de Catanzaro et al., 1996) and signaling pheromones (Kannan et al., 1998; Kannan and Archunan, 2001; Zhang et al., 2008). The female rats show strong preference towards the normal male preputial gland secretion as compared to that of castrated rats. This confirms the presence of sex pheromone(s) in the preputial gland and is controlled by testosterone (Orsulak and Gawienowski, 1972; Novotny et al., 1990; Jemiolo et al., 1991; Zhang et al., 2008).

In several mammals, volatile pheromones are associated with soluble binding protein belonging to the family of lipocalins (Cavaggioni et al., 1990; Marchese et al., 1998; D’Innocenzo et al., 2006). Lipocalins (17-20 kDa) include the fatty acid binding proteins, avidins, a group of metalloproteinase inhibitors and triabin. This super family is characterized by a similar structure, a repeated +1 topology of β-barrel, and by the conservation of a remarkable structural signature (Flower et al., 2000). Lipocalins are structurally heterogenous proteins in animals, plants and bacteria (Flower et al., 2000; Grzyb et al., 2006). They comprise more than 100 relatively small proteins secreted in various body fluids (Pervaiz and Brew, 1987).

Members of the lipocalin family share a common motif, and are characterized by a similar structural framework consisting of eight anti-parallel β-strands, that fold to form a β-barrel, and an α-helical domain located near the carboxy terminal (Cavaggioni and Mucignat-Caretta, 2000). Inside the β-barrel lies the ligand binding site. The structure of this barrel allows the non-covalent binding small apolar molecules such as lipids, steroids and odorants and therefore is particularly suited for pheromonal communication (Grzyb et al., 2006). The pheromone carrying proteins are reported in mouse urine (Finlayson et al., 1965; Robertson et al., 1993), rat urine (Dinh et al., 1965; Cavaggioni and Mucignat-Caretta, 2000), boar saliva (Marchese et al., 1998), hamster vaginal secretion (Singer et al., 1986), human sweat (Zeng et al., 1996) and horse sweat (D’Innocenzo et al., 2006). These proteins are belonging to lipocalin family and exhibits significant sequence similarity with vertebrate odorant binding proteins (OBPs) (Pelosi, 1994, 1996).
In rodents, attractiveness of males correlates positively with secretion of androgens, which affects scent marking behaviour, production of sexual pheromones and the concentration of pheromone carrying protein (Ferkin et al., 1994; Churakov and Novikov, 2000). In attempts to identify the active compounds in the excretions or secretions used for pheromonal communication, a dependence of the animal products on the endocrine system for regulation of their chemical composition can be experimentally advantageous. For example, if the pheromonal activity of an excretory or secretory products varies in relation to the endocrine physiology of an animals that produce it, an examination of constituents whose presence in the product exhibits a parallel variation could provide clues to identify the active compounds subject to further verification of their activities via biological assays (Macrides et al., 1984; Novotny et al., 1999a,b; Zhang et al., 2007a). This methodology for exploring pheromones in mammals has led to success in the discovery of a number of pheromones in the house mouse, *Mus musculus* (Novotny et al., 1999b; Schwende et al., 1986; Zhang et al., 2007a), laboratory mouse (Achiraman and Archunan, 2005), voles (Zhang et al., 2007b) and rat (Zhang et al., 2008). Similarly endocrine dependency of pheromone carrying proteins has been reported in rat (Kamalakkannan et al., 2006), mice (Cavaggioni and Muciganat Caretta, 2000) and boar (Marchese et al., 1998).

Among the scent glands reported in rodents, preputial glands are often well developed in males than in females and are regulated by androgen and suppressed by castration. Hence, the present study focused on the identification of endocrine dependent volatile compounds and the major low molecular mass proteins in the preputial gland of rat.
1.2. MATERIALS AND METHODS

1.2.1. Animal

Adult male and female laboratory rats (Wistar strain), Rattus norvegicus procured from Indian Institute of Science, Bangalore, India, were allowed to mate in the animal room; their litters were reared under laboratory conditions (light on from 06.00 to 18.00 h, temperature 24±1°C) and fed with rat feed (Sai Durga feeds, Bangalore) and water ad libitum. The bedding material was changed twice a week.

1.2.2. Castration

Reproductively active male rats were anesthetized with Pentothal sodium (Abbot Lab Ltd., India) at the rate of 60 mg/kg of body weight dissolved in saline and injected intra-peritonially. Hairs on the lower abdomen were removed gently. An oblique incision, half an inch in length, was made to expose the muscle layer. By gently squeezing the scrotal sacs, the testes were removed; the muscle and fat tissues were pushed into the scrotum and the skin was stitched before treatment with safromycin. Castrated male rats were maintained under normal conditions for about four weeks.

1.2.3. Hormone replacement

Testosterone enthalate (testoviron; Schering, Germany) was diluted in arachis oil and administered intramuscularly to castrated rat at a rate of 10 mg/day/ rat for 2 weeks. One milliliter of testoviron contained 250 mg of testosterone enthalate. 1 ml of testosterone was made up to 25 ml by adding 24 ml of arachis oil. From this, 1 ml was taken and administered intramuscularly to six castrated males. After the scheduled period of testosterone supplementation, the animals were allowed for incubation for two weeks.

1.2.4. Testosterone Assay

After all injections, serum was separated from blood after centrifugation and samples were stored at -20°C. Testosterone was estimated in blood samples using solid phase (antibody coated tubes) RIA system wherein the antibody was immobilized on
the wall of the tubes. This method was based on the competition of unlabelled testosterone in the standard or samples of \([\text{I}^{125}]\) testosterone for the binding sites on the antibody coated tubes. They were allowed to reach equilibrium at the end of incubation period. The bound antibody and free antigens were separated by decanting the tubes. Testosterone concentration of sample was quantified by measuring the radioactivity associated with bound fraction of samples and standards (Sufi et al., 1986).

**Reagents**

1. **Buffered \([\text{I}^{125}]\) testosterone**
   
   One vial of iodinated testosterone with a specific activity of 3 uci or 111 K bq was provided.

2. **Testosterone calibrations**

   Testosterone standards of 0, 20, 50, 150, 500, 1800 and 3600 ng/ml were used as calibrators to plot standard graph for reference.

**Procedure**

The assay was carried out in duplicate with total, non-specific binding, maximum binding, standards, quality controls and serum sample tubes.

1. Plain 12 X 75 mm polypropylene tubes in duplicate were used for total and non specific binding.

2. Testosterone antibody coated polypropylene tubes were used for maximum binding, standards and serum samples.

Reagents were added as follows

1. **Total count tubes**

   1 ml of buffered \([\text{I}^{125}]\) testosterone

2. **Non-specific binding**

   1 ml of buffered \([\text{I}^{125}]\) testosterone tubes (NSB)
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3. Maximum binding tubes

1 ml of buffered [I\textsuperscript{125}] testosterone (antibody coated tubes)

4. Standard tubes

100 µl of different standards (20-3600 ng/ml) and 1 ml of buffered [I\textsuperscript{125}] testosterone

5. Unknown serum tubes

100 µl of serum samples and 1 ml of buffered [I\textsuperscript{125}] testosterone

Tubes were agitated and vortexed gently. They were incubated for 3 h at 37° C. At the end of the incubation, all tubes were thoroughly decanted (except total count tubes) and counted for one minute in a microprocessor based LKB Rack Gamma counter. Testosterone administration in a sample was calculated from a logit-log representation of the calibration curve and expressed as ng/ml.

The sensitivity of the assay was 5 ng/ml using 3 h room temperature procedure. The maximum binding was 35% to 45%. The antiserum was highly specific for testosterone with very low cross reactivity to other compounds that might be present in the serum samples.

1.2.5. Excision of preputial glands

Institutional Animal Ethics Committee (IAEC), established under the auspices of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Bharathidasan University, Tiruchirappalli approved the experiment for animal use. Six male pups (15-20 days), six adult intact males (3 months), six castrated males and six castrated with testosterone treated rats were subjected to cervical dislocation. Following autopsy, preputial gland was removed and frozen immediately at -20°C until use.
1.2.6. **Histology**

The histology of preputial gland was performed by adopting the routine paraffin method (Humason, 1979).

**Reagents**

*Physiological saline (0.9%):* 0.9g of sodium chloride in 100 ml distilled water.

*Bouin's fluid:* prepared by adding 75 ml of saturated picric acid, 25 ml of formaldehyde and 5 ml of glacial acetic acid and filtered.

*Harris hematoxylin:* It was prepared by dissolving 1g of hematoxylin in 10 ml of absolute alcohol. 20 g of ammonium alum, previously dissolved in 200 ml of hot water was added to the above. This solution was quickly boiled and 0.5 g of mercuric oxide was added to it upon which the solution turned to dark purple color. It was cooled rapidly under the tap and filtered before use. 8 ml of glacial acetic acid was added after cooling to sharpen nuclear staining.

*Eosin:* 0.5 g of water soluble eosin (w.s) powder was dissolved in 30 ml of distilled water and made up to 100 ml with 70 ml of alcohol.

**Procedure**

Preputial gland was dissected out from the male rats, were blotted free of blood and mucus, washed thoroughly in physiological saline, cut into pieces of desired size and fixed in Bouin’s fluid fixative immediately after autopsy. Fixation was carried out at room temperature for 24 h, after which the tissues were transferred to 70% alcohol. Several changes of 70% alcohol were given until the yellow color disappeared from the tissues. The tissues were then dehydrated by passing through ascending grades of alcohol, cleared in xylene, infiltrated with molten paraffin, and finally embedded in paraffin wax (58°C M.P).
Transverse and longitudinal sections with 3-5 μm thickness were obtained using a rotary microtome (Leica, Germany). The sections, thus obtained, were stained in Harris hematoxylen and eosin, dehydrated using alcohol, cleared in xylene and mounted using DPX. The stained slides were observed in a Carl Zeiss (Germany) Axio 2 plus research microscope. Images were captured through a CCD camera in a computer.

1.2.7. Estimation of proteins

**Extraction buffer (Phosphate buffer saline- pH 7.2)**

- Monosodium dihydrogen phosphate (NaH$_2$PO$_4$) - 780 mg
- Disodium monohydrogen phosphate (Na$_2$HPO$_4$) - 709 mg
- Sodium chloride (NaCl) - 876 mg

- Dissolved in 75 ml of distilled water.
- pH was adjusted with HCl and the volume was made up to 100 ml.

**Preparation of extracts**

The crude extract from the preputial gland was prepared by homogenization with Phosphate Buffer Saline (pH 7.2) in well-sterilized homogenizer under ice-cold condition, followed by centrifugation at 10,000 rpm for 15 min. The clear supernatant was collected and the protein present in the supernatant was determined adapting the method of Bradford (1976) using Bovine Serum Albumin (BSA) as standard.

**Principle**

Coomassie Brilliant Blue G250 was used for quantification of protein in solution. The dye and the protein form a complex by a non-covalent bond to form the non-covalent compound. The intensity of the protein-dye complex was measured at 595 nm in a spectrophotometer.
Reagents

Bradford reagent: 100 mg of Coomassie Brilliant Blue G250 was dissolved in 50 ml of 95% ethanol and to this 100 ml of 85% orthophosphoric acid was added. The resultant solution was diluted to a final volume of 1 litre.

Procedure

The standard bovine serum albumin (BSA) solution (stock solution = 1 mg/ml) of varying series of concentration (10 μg to 100 μg) was taken and made up to 1 ml using distilled water in test tubes. The unknown samples, 1-5 μl, were taken and then made up to 1 ml with distilled water. To all the tubes, 2.5 ml of Bradford’s reagent was added. After 2 min., optical density (O.D) was measured at 595 nm using ultra violet-visible (UV-vis) spectrophotometer (Perkin-Elmer, USA) and standard graph was prepared, where X-axis represented the concentration of standard and O.D was plotted in the Y-axis. From this graph, the protein concentration of unknown sample was calculated and the volume to be loaded in the SDS-PAGE was decided upon.

Calculation of protein concentration

\[
\text{Concentration of protein in the sample} = \frac{\text{Concentration of the standard}}{\text{Optical density of the standard}} \times \frac{\text{Optical density}}{	ext{Volume of sample taken (μl)}}
\]

\[
\text{Volume to be loaded} = \frac{\text{Volume of sample taken (μl)}}{\text{Protein concentration in sample (X μg)}} \times 50 \, \mu\text{g}
\]

1.2.8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970)

Principle

SDS-PAGE is one of the most common methods adopted to fractionate polypeptides on the basis of their molecular sizes in electric fields. Depending upon the concentration of the acrylamide, the pore size will favour protein separation. The
acrylamide concentration is inversely proportional to pore size of the gel. The ionic
detergent SDS helps to eliminate secondary structure of the polypeptide and impart
negative charge on proteins so that all fractions of polypeptide move towards the anode
for free resolution. The mobility of the peptide in SDS-PAGE is directly proportional to
its molecular size and the applied electric field.

Reagents required

i. Monomer: Acrylamide solution (30%)
   Acrylamide - 14.6 g
   Bisacrylamide - 0.4 g
   Water make up to 50 ml (store at 4°C in dark room)

ii. Running gel buffer (lower tris) (pH-8.8)
   1.5 M Tris-buffer (MW-121.14) - 9.075g
   Adjust pH with HCl
   Water make up to 50 ml.

iii. Stacking gel buffer (upper tris) (pH-6.8)
   1M Tris-buffer (MW-121.14) - 3.029g
   Adjust pH with HCl
   Water make up to 25 ml.

iv. 10% Sodium Dodecyl Sulphate (SDS)
   SDS - 2.5 g
   Water make up to 25 ml.

v. 10% Ammonium per sulphate (APS)
   APS - 0.5 g
   Water make up to 5 ml.
vi. **Tank buffer (1x) (pH - 8.2)**

- Tris buffer (molecular weight -121.4) - 750 mg
- Glysine - 3.6 g
- SDS - 250 mg

Distilled water make up to 250 ml.

vii. **Sample buffer (1x)**

- Tris - 1.25 ml of stacking gel buffer (pH 6.8)
- SDS - 2.0 ml solution of 10% SDS
- Glycerol - 1 ml
- β-2-mercaptoethanol - 0.5 ml

A pinch of bromophenol blue was added as indicator. Water make up to 5 ml.

viii. **Staining solution: (Coomassie Brilliant Blue R-250) (0.5% stain)**

- 45% methanol - 50 ml
- 10% acetic acid - 10 ml
- Coomassie R-250 - 500 mg

Distilled water make up to 100 ml.

ix. **Destaining solution**

- 50% methanol - 50 ml
- 10% acetic acid - 10 ml

Distilled water make up to 100 ml.

The gel was prepared by mixing the stock solution mentioned previously.

x. **Separating gel (12%)**

- Dis. Water - 3.3 ml
- 30% acrylamide - 4 ml
- Tris (pH 8.8) - 2.5 ml
- 10% SDS - 0.1 ml
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xi. **Stacking gel (5%)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dis. Water</td>
<td>2.1 ml</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Tris (pH 6.8)</td>
<td>0.38 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.03 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.03 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.003 ml</td>
</tr>
</tbody>
</table>

**Procedure**

The PAGE apparatus was assembled in which two glass plates were sandwiched using 1.5 mm space strip and the bottom was sealed with 1% agar. A 12% separating gel was prepared and poured between the two glass plates and was allowed for polymerization. A few drops of separating gel-overlying solution was layered over the separating gel and decanted after 30 s. Then the 5% stacking gel was poured on top of the separating gel and the comb was inserted and removed gently after solidification and the wells were rinsed with distilled water. The plate was then dispensed in both chambers of the apparatus.

For the separation, 50 μg protein of each sample was loaded on the gel for comparative studies. The supernatant were boiled in equal volume of sample buffer and were loaded onto wells of the gel. For determination of molecular weight, 4 μl of protein standard (Protein Molecular Weight Marker-Medium range, Genei, Bangalore), was applied on the gel. Initially an electric current of 50 V was applied till the dye entered the separating gel. Subsequently the electric current was increased to 100 V till the tracking dye reached just 2 to 3 mm above the bottom of the gel. After electrophoresis, the gel was removed from the glass plate and the resolved peptides were revealed by Coomassie Brilliant Blue R-250 staining solution.
The molecular weight of the unknown polypeptides in the samples were identified using gel documentation scanner and analysis software (Quantity One, BioRad, CA, USA).

1.2.9. Extraction of volatiles

Following autopsy, preputial glands were removed and placed in dichloromethane and ground well with glass homogenizer under ice-cold condition. Immediately after homogenization, the supernatant was filtered through silica gel (50-60 μ mesh size). The supernatant was collected in a glass vial and sealed with air tight screw cap vials. The sample vials were stored at -20°C until the analysis.

1.2.10. Gas chromatography-Mass spectrometry (GC-MS)

Principle

The Gas Chromatography/Mass Spectrometry (GC/MS) instrument separates chemical mixtures (the GC component) and identifies the components at the molecular level (the MS component). It is one of the most accurate tools for analyzing environmental samples. The GC works on the principle that a mixture will separate into individual substances when heated. The heated gases are carried through a column with an inert gas (such as helium). As the separated substances emerge from the column opening, they flow into the MS. Mass spectrometry identifies compounds by the mass of the analyte molecule. A “library” of known mass spectra, covering several thousand compounds, are stored on a computer. Mass spectrometry is considered as the only definitive analytical detector.

Procedure

The GC-MS analyses were made in QP-2010 (Shimadzu, Japan). 1 μl of extract was injected into GC-MS on a 30 m DB-1 capillary column with a film thickness of 0.25 μm (30 m X 0.32 mm i.d. coated with DB-1 using the following temperature programme, initial oven temperature of 70°C for 35 min. The gas chromatography was equipped with Quadrupole detector. The GC-MS was under the computer control.
at 70 eV. Identification of unknown compounds was made by probability based matching using the computer library built within WILEY7, NIST05 and NIST05s.

GC-MS programme

Name of GC-MS: GC-MS QP 2010 (SCHIMADZU, JAPAN)

**GC-parameters**

- **Column Oven Temperature**: 70.00°C
- **Injection Temperature**: 250.00°C
- **Injection Mode**: Splitless
- **Sampling Time**: 3.00 min
- **Flow Control Mode**: Linear Velocity
- **Pressure**: 18.8 kPa
- **Total Flow**: 30.0 ml/min
- **Column Flow**: 1.47 ml/min
- **Linear Velocity**: 44.4 cm/sec
- **Purge Flow**: 3.0 ml/min
- **Split Ratio**: 17.4
- **Ion Source Temperature**: 230.00 °C
- **Interface Temperature**: 270.00 °C
- **Solvent Cut Time**: 3.00 min
- **Detector Gain Mode**: Relative
- **Detector Gain**: 0.10 kV
- **Threshold**: 500

**MS-Parameters**

- **Start Time**: 5.00 min
- **End Time**: 35.00 min
- **ACQ Mode**: Scan
- **Event Time**: 0.50 sec
- **Scan Speed**: 10.00
1.2.11. Statistical Analysis

The Duncan’s Multiple Rank Test (DMRT) was used to compare the body and preputial gland weight, serum testosterone level and total protein content across the intact, castrated, prepubertal and castrated with testosterone treated rats.

1.3. RESULTS

1.3.1. Effect of castration on the morphology of preputial gland

Preputial gland was shown to undergo shrinkage following castration and the testosterone therapy restored the glandular size (Fig. 1.1).

1.3.2. Preputial gland weight and serum testosterone level

The influences of castration and hormone replacement on preputial gland weight and serum testosterone level are summarized in Table 1.1. Castration led to the significant loss of glandular weight in the rat. Further, it was slightly higher than the prepubertal preputial gland, whereas testosterone administration resulted in a marked level of increase in the glandular weight when compared to the castrated preputial gland.

Testosterone level in the serum of intact males was 470.83±2.44 ng/dl. By contrast, the testosterone level in castrated males was very low 12.49±1.33 ng/dl. However, the level of testosterone in testosterone treated castrated males (520.6±2.07 ng/dl) was higher than castrated males (12.49±1.33 ng/dl). The prepubertal male showed low level of testosterone (10.33±2.05 ng/dl) comparable to that seen in castrated males.
1.3.3. **Histological variations**

The preputial glands of the rat are holocrine, branched tubulo-alveolar glands. Developmentally and functionally they are modified sebaceous glands. The microscopic observation of intact preputial gland showed that the preputial gland parenchyma consisted of acini (acinar tissue) and was lobulated by thin connective tissue (Fig. 1.2). The castrated and prepubertal male preputial gland exhibited totally disorganized typical acinus tissue structure. The reorganization of acinar cells was observed in the castrated with testosterone-treated rats.

1.3.4. **Protein concentration**

The concentrations of total protein in the preputial gland of castrated (31.22±0.702 mg) and prepubertal (31.34±0.720 mg) were significantly lower than the intact preputial gland (44.00±0.626 mg) (Table 1.2). More interestingly, similar response was observed in the preputial gland of castrated rat and prepubertal rat. However, the protein content in preputial gland of castrated rats was reinstated by testosterone treatment (46.09±0.582 mg).

1.3.5. **SDS PAGE analysis**

The protein profiles of preputial gland of intact rat was compared to that of castrated, prepubertal and castrated with testosterone treated rats. In all these groups, the preputial gland invariably exhibited 12 polypeptides in Coomassie Brilliant Blue stained gel; molecular mass ranged between 18 and 123 kDa (Fig. 1.3). The staining intensity of all major polypeptides was found to be similar in preputial gland of intact and castrated with testosterone treated rats. However, the number and staining intensity of polypeptides in the preputial gland of castrated rat as well as prepubertal rats were found to be altered when compared to intact rat preputial gland. By contrast, the staining intensity of polypeptide with molecular mass of 63 kDa was found to be higher in the preputial gland of castrated and prepubertal rats. The protein profiles of castrated and prepubertal rats showed similar banding patterns. Two polypeptides namely 21.0
kDa and 34.0 kDa were absent in prepubertal and castrated rats. These observations were confirmed by the band areas determined by densitometric scanning (Fig. 1.4).

The present study mainly focused on the identification of major low molecular mass proteins in the rat preputial gland and their endocrine dependency. The electrophoretic profile showed high intensity of 18 kDa mass protein in the intact rat preputial gland. On castration, the intensity of this protein was greatly reduced which was similar to prepubertal male rat preputial gland. The increasing intensity of 18 kDa and other proteins was observed after the administration of testosterone in the castrated rats (Fig. 1.5).

1.3.6. Volatile compounds

The gas chromatography analysis clearly showed that the major compounds fell between the retention times of 5 and 35 min. The gas chromatogram and the identified compounds in the preputial gland of intact, castrated, prepubertal and castrated with testosterone treated rats are shown in Fig. 1.6 and Tables 1.3 and 1.4. The computer-matched data of the identified compounds showed greater than 95% similarity with the compounds identified from preputial gland extracts. Totally, 31 different volatile compounds were identified in intact rat preputial gland. Among these, 5 compounds namely geranyl linalool isomer, oxirane, farnesol, tetratriacontane and lanosterol were absent in castrated rat preputial gland. The compounds like hexadecyl ester decanoic acid, 9 octadecanoic(Z)-tetradecyl ester and octadecylester octadecanoic acid were appeared only in preputial gland of castrated rats, which are considered as testosterone independent. On testosterone administration, there was a reappearance of missing compounds in castrated rats except tetratriacontane. The unique compounds which were present only in the preputial gland of castrated rat disappeared after testosterone replacement. While 31 compounds were identified in intact rat preputial gland, the prepubertal preputial gland showed 22 compounds. The remaining 9 compounds such as 3-bromocholest-5-ene, 3,7,11,15-tetramethyl-1,6,10,14-hexadecatetraen-3-ol, geranyl linalool isomer, oxirane, farnesol, tetratriacontane, cholestan-3-ol, cholest-7-en-
Fig. 1.1. Effect of castration on the morphology of the preputial gland
(A - intact adult male; B - castrated adult male; C - prepubertal male;
D - castrated with testosterone treated male)
Fig. 1.2. Histological variations in the rat preputial gland of various groups (H&E)
(LO-Location of preputial gland 50x; INT-Intact 200x; CAS-Castrated 250x; PUP- Prepuberta. 230x; CAS+T-Castrated with testosterone treated 250x)
Fig. 1.3. Electrophoretic distribution of total proteins; Lanes 1, 2, 3, 4 and 5 represent molecular weight marker, extracts from preputial gland of intact male, castrated male, prepubertal male, castrated with testosterone treated male rats respectively.
Fig. 1.4. Densitometric pattern of preputial glandular protein profiles of rats.

Fig. 1.5. Densitometric pattern of 18 kDa protein in preputial gland of rats.
Fig. 1.6. Comparison of the preputial gland volatile chromatograms of rats
(For peak numbers, refer to tables 1.3 & 1.4)
Table 1.1. Effect of castration on body and organ weight and serum testosterone level in rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Intact adult male</th>
<th>Castrated male</th>
<th>Prepubertal male</th>
<th>Castrated with hormone treated male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>158±2.00</td>
<td>195.83±5.71\textsuperscript{ab}</td>
<td>36.66±0.88\textsuperscript{d}</td>
<td>204.16±3.52\textsuperscript{a}</td>
</tr>
<tr>
<td>Preputial gland weight (mg)</td>
<td>125.18±5.20\textsuperscript{b}</td>
<td>59.17±8.73\textsuperscript{e}</td>
<td>19.23±2.31\textsuperscript{d}</td>
<td>167.63±6.30\textsuperscript{a}</td>
</tr>
<tr>
<td>Testosterone Concentration (ng/dl)</td>
<td>470.83±2.44\textsuperscript{b}</td>
<td>12.49±1.33\textsuperscript{e}</td>
<td>10.33±2.05\textsuperscript{cd}</td>
<td>520.60±2.07\textsuperscript{a}</td>
</tr>
</tbody>
</table>

Each value represents ± SE of 6 observations.
Means within a column followed by the same letters are not significant at \( p<0.05 \) according to DMRT

Table 1.2. Quantification of proteins in rat preputial glands

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Test animals</th>
<th>Total Protein (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Intact adult male</td>
<td>44.00±0.626\textsuperscript{ab}</td>
</tr>
<tr>
<td>2</td>
<td>Castrated male</td>
<td>31.22±0.702\textsuperscript{b}</td>
</tr>
<tr>
<td>3</td>
<td>Prepubertal male</td>
<td>31.34±0.720\textsuperscript{b}</td>
</tr>
<tr>
<td>4</td>
<td>Testosterone treated castrated male</td>
<td>46.09±0.582\textsuperscript{a}</td>
</tr>
</tbody>
</table>

Each value represents ± SE of 6 observations.
Means within a column followed by the same letters are not significant at \( p<0.05 \) according to DMRT
Table 1.3. List of volatile compounds identified in preputial gland of rat in various experimental groups (I-intact, C-castrated, C+T-castrated with testosterone treated and P-prepubertal rat)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Compounds</th>
<th>I</th>
<th>C</th>
<th>C+T</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4-ethyl decane</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Heptadecane</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Tetradecane</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>2,6,11-trimethyl dodecane</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Octadecane</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>3,7-dimethyl decane</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Nonadecane</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>4-methyl tetradecane</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Docosane</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>1,1,2,3,3-pentachloropropane</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>Hexadecane</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>2-methyl pentadecane</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>Eicosane</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>Octacosane</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>Pentachlorobutene</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>3,5-cyclo-6,8(14),22-ergostatriene</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>Squalene</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>3-bromocholesterol-5-ene</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>---</td>
</tr>
<tr>
<td>19</td>
<td>3,7,11,15-tetramethyl-1,6,10,14-hexadecatetraen-3-ol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>---</td>
</tr>
<tr>
<td>20</td>
<td>Geranyl linalool isomer</td>
<td>+</td>
<td>---</td>
<td>+</td>
<td>---</td>
</tr>
<tr>
<td>21</td>
<td>Oxirane</td>
<td>+</td>
<td>---</td>
<td>+</td>
<td>---</td>
</tr>
<tr>
<td>22</td>
<td>Farnesol</td>
<td>+</td>
<td>---</td>
<td>+</td>
<td>---</td>
</tr>
<tr>
<td>23</td>
<td>Tetratriacontane</td>
<td>+</td>
<td>---</td>
<td>+</td>
<td>---</td>
</tr>
<tr>
<td>24</td>
<td>2,6,11,15-tetramethyl-hexadeca-2,6,8,10,14-pentene</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>---</td>
</tr>
<tr>
<td>25</td>
<td>Cholest-5-en-3-ol (3 beta)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>26</td>
<td>Cholestan-3-ol</td>
<td>+</td>
<td>+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>27</td>
<td>Cholest-7-en-3-ol</td>
<td>+</td>
<td>+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>28</td>
<td>Lanosterol</td>
<td>+</td>
<td>---</td>
<td>+</td>
<td>---</td>
</tr>
<tr>
<td>29</td>
<td>Hexadecyl ester hexa decanoic acid</td>
<td>---</td>
<td>+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>30</td>
<td>Tetrakis(2,3-ditert-butyl phenyl)4-4-biphenylene diphosphonate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>31</td>
<td>9 octadecanoic (Z) -tetradecyl ester</td>
<td>---</td>
<td>+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>32</td>
<td>Octadecanoic acid, octadecyl ester</td>
<td>---</td>
<td>+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>33</td>
<td>2-tert-butyl-4,6-bis (3,5-di-tert-butyl-4-hydroxybenzyl)phenol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>34</td>
<td>Dodecanoic acid, 1,2,3-propanetrieyl ester</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 1.4. Molecular weight and molecular formula of identified compounds in the preputial gland of rats

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Compounds</th>
<th>Mol. Wt</th>
<th>Mol. For.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4-ethyl decane</td>
<td>170</td>
<td>C_{12}H_{26}</td>
</tr>
<tr>
<td>2</td>
<td>Heptadecane</td>
<td>240</td>
<td>C_{17}H_{36}</td>
</tr>
<tr>
<td>3</td>
<td>Tetradecane</td>
<td>198</td>
<td>C_{14}H_{30}</td>
</tr>
<tr>
<td>4</td>
<td>2,6,11-trimethyl dodecane</td>
<td>212</td>
<td>C_{15}H_{32}</td>
</tr>
<tr>
<td>5</td>
<td>Octadecane</td>
<td>254</td>
<td>C_{18}H_{38}</td>
</tr>
<tr>
<td>6</td>
<td>3,7-dimethyl decane</td>
<td>170</td>
<td>C_{12}H_{26}</td>
</tr>
<tr>
<td>7</td>
<td>Nonadecane</td>
<td>268</td>
<td>C_{19}H_{40}</td>
</tr>
<tr>
<td>8</td>
<td>4-methyl tetradecane</td>
<td>212</td>
<td>C_{15}H_{32}</td>
</tr>
<tr>
<td>9</td>
<td>Docasane</td>
<td>310</td>
<td>C_{22}H_{46}</td>
</tr>
<tr>
<td>10</td>
<td>1,1,2,3,3-pentachloro propane</td>
<td>214</td>
<td>C_{3}H_{3}Cl_{5}</td>
</tr>
<tr>
<td>11</td>
<td>Hexadecane</td>
<td>226</td>
<td>C_{16}H_{34}</td>
</tr>
<tr>
<td>12</td>
<td>2-methyl pentadecane</td>
<td>226</td>
<td>C_{16}H_{34}</td>
</tr>
<tr>
<td>13</td>
<td>Eicosane</td>
<td>282</td>
<td>C_{20}H_{42}</td>
</tr>
<tr>
<td>14</td>
<td>Octacosane</td>
<td>394</td>
<td>C_{28}H_{58}</td>
</tr>
<tr>
<td>15</td>
<td>Pentachlorobutene</td>
<td>226</td>
<td>C_{4}H_{3}Cl_{5}</td>
</tr>
<tr>
<td>16</td>
<td>3,5,cyclo-6,8 (14),22-ergostatriene</td>
<td>378</td>
<td>C_{28}H_{42}</td>
</tr>
<tr>
<td>17</td>
<td>Squalene</td>
<td>410</td>
<td>C_{30}H_{50}</td>
</tr>
<tr>
<td>18</td>
<td>3-bromocholeste-5-ene</td>
<td>368</td>
<td>C_{27}H_{44}</td>
</tr>
<tr>
<td>19</td>
<td>3,7,11,15-tetramethyl-1,6,10,14-hexadecatetraen-3-ol</td>
<td>290</td>
<td>C_{20}H_{34}O</td>
</tr>
<tr>
<td>20</td>
<td>Geranyl linalool isomer</td>
<td>426</td>
<td>C_{20}H_{34}O</td>
</tr>
<tr>
<td>21</td>
<td>Oxirane</td>
<td>426</td>
<td>C_{30}H_{50}O</td>
</tr>
<tr>
<td>22</td>
<td>Farnesol</td>
<td>222</td>
<td>C_{15}H_{26}O</td>
</tr>
<tr>
<td>23</td>
<td>Tetraatriacontane</td>
<td>478</td>
<td>C_{34}H_{70}</td>
</tr>
<tr>
<td>24</td>
<td>2,6,11,15-tetramethyl-hexadeca-2,6,8,10,14-pentene</td>
<td>272</td>
<td>C_{20}H_{32}</td>
</tr>
<tr>
<td>25</td>
<td>Cholest-5-en-3-ol (3 beta)</td>
<td>386</td>
<td>C_{27}H_{46}O</td>
</tr>
<tr>
<td>26</td>
<td>Cholestan-3-ol</td>
<td>388</td>
<td>C_{27}H_{46}O</td>
</tr>
<tr>
<td>27</td>
<td>Cholest-7-en-3-ol</td>
<td>386</td>
<td>C_{27}H_{46}O</td>
</tr>
<tr>
<td>28</td>
<td>Lanosterol</td>
<td>426</td>
<td>C_{30}H_{50}O</td>
</tr>
<tr>
<td>29</td>
<td>Hexadecyl ester hexa decanoic acid</td>
<td>480</td>
<td>C_{32}H_{64}O _2</td>
</tr>
<tr>
<td>30</td>
<td>Tetrakis(2,3-ditert-butyl phenyl)4-4-biphenylene diphosphonate</td>
<td>1038</td>
<td>C_{68}H_{95}O_{4}P_{2}</td>
</tr>
<tr>
<td>31</td>
<td>9 octadecanoic (Z)–tetradecyl ester</td>
<td>478</td>
<td>C_{32}H_{62}O_{2}</td>
</tr>
<tr>
<td>32</td>
<td>Octadecanoic acid, octadecyl ester</td>
<td>507</td>
<td>C_{34}H_{66}O_{2}</td>
</tr>
<tr>
<td>33</td>
<td>2-tert-butyl-4,6-bis (3,5-di-tert-butyl-4-hydroxybenzyl)pheno</td>
<td>586</td>
<td>C_{40}H_{50}O_{3}</td>
</tr>
<tr>
<td>34</td>
<td>Dodecanoic acid, 1,2,3-propanetriethyl ester</td>
<td>639</td>
<td>C_{39}H_{74}O_{6}</td>
</tr>
</tbody>
</table>
3-ol and lanosterol are supposed to be adult-specific. Notably, the testosterone dependent compounds were exclusively absent in the preputial gland of prepubertal rat.

1.4. DISCUSSION

These results demonstrate that castration affects the morphology of preputial gland, weight, histology, total protein content, protein profiles and volatile compounds. It is well known that testosterone is known to influence the development of skin glands in rodents (Kannan and Archunan, 1998; Kamalakkannan et al., 2006; Zhang et al., 2007a,b, 2008; Pohorecky et al., 2008).

The testosterone dependency of preputial gland is consistent with previous work in a sense that castration can lead to atrophy of preputial gland of mouse, vole and rat (Jannet, 1978; Brinck and Hoffmeyer, 1984; Zhang et al., 2007b, 2008). It is also reported that dominant males have higher testosterone levels than subordinates and there is an increase in preputial glandular weight when male attains dominance (McKinney and Pasley, 1973; Bronson and Marsden, 1973; Rowsemtt et al., 1988). Similar kinds of results were also obtained from other species. For instance, Kennaugh et al. (1977) reported an enlargement of preputial gland of male fallow deer in the reproductive season. In male Japanese serows, Sawada et al. (1987) observed a significant increase in the preputial gland in winter and suggested that the gland probably grows in the breeding season (autumn) owing to an active secretion and thereafter declines in winter because of an inactivation of cell division; the sebaceous gland in goat skin enlarged during the testosterone treatment and caused male effect but reduced in size after the removal of testosterone (Iwata et al., 2000). These results suggest that development and secretory activity of preputial gland are associated with testosterone. It is assumed that changes in glandular morphology would definitely change the histoarchitecture, protein profiles and volatile compounds.

It is well known that preputial glands are modified sebaceous glands. The main difference between the modified sebaceous gland and the ordinary sebaceous gland is
the presence of broad differentiating cell layer and the continuous maturation of small to large lipid droplets therein (Atoji et al., 1989). It is reported that secretions from the sebaceous glands act as a vehicle for the volatile substances (Kannan and Archunan, 1998). The changes in the histoarchitecture of preputial gland are in agreement with several reports. For example, gonadectomy reduces the weight of the preputial glands, depressing their sebum content and producing degenerative structural changes (Ebling, 1963; Homady, 1982). Testosterone has been shown to stimulate growth, differentiation and secretion, producing hypertrophy of the parenchymatous cells and increasing ductular formation in the preputial gland of long term castrated mice (Brain and Homady, 1985). Similarly, Beaver (1960) found that the administration of testosterone to new born and weaning animals resulted increase in the number of mitosis in the basal cells, increased lipogenesis, acinar genesis and increased ductular formation and maturation was hastened. It indicates that testosterone causes the maturation of preputial gland and increases the sebum secretion containing volatile compounds.

Soluble proteins of low molecular mass seem to play an important role in chemical communication (Pelosi, 1994). They are present at high concentrations in biological fluids involved both in perception and delivery of chemical messages of pheromonal significance. In the present study, total protein content and the intensity of major low molecular mass protein, 18 kDa protein, in the preputial gland were significantly decreased in castrated rats and similar to prepubertal rat. It is due to the removal of testes which depleted the testosterone level and caused significant changes in the protein profiles of the preputial gland. In fact, the intensity of the band from castrated rat preputial gland was rather less when compared to that obtained in normal male rats.

The presence of major low molecular mass protein (18 kDa) in the preputial gland is consistent with the earlier reports of presence of pheromone carrying proteins with the molecular weight of 17-20 kDa in the urine of mouse (Robertson et al., 1993)
and rat (Dinh et al., 1965), saliva of pig (Marchese et al., 1998; Cavaggioni and Mucignat-Caretta, 2000), vaginal fluid of hamster (Singer et al., 1986) and preputial gland of house rat (Archunan et al., 2004). Interestingly, odorant binding proteins (OBPs) of vertebrates have the molecular weight of around 20 kDa (Pelosi, 1994). Further, the testosterone dependency of the low molecular mass protein gains support from the earlier report of androgen dependency of proteins in the preputial gland of house rat (Rattus rattus) (Kamalakkannan et al., 2006). Similar observation was made in the urine of mice. Indeed, the testosterone induces the synthesis of major low molecular mass protein i.e., Major Urinary Protein (MUP) in adult female mice (Cavaggioni and Mucignat-Caretta, 2000). The castrated boar failed to express the salivary lipocalin (20 kDa) in the salivary gland and reconstituted after testosterone supplementation (Marchese et al., 1998). In female hamster, aphrodisin (17 kDa) is secreted in vaginal discharge due to surge of estrogen and facilitate to attract males. All these proteins are reported as pheromone binding proteins and carrying the sex pheromones. As preputial gland plays a significant role in pheromonal communication, the presently identified 18 kDa may have a role for pheromone communication. It is likely that this protein may belong to lipocalin family and probably carry the sex pheromones produced by preputial gland.

Identification of endocrine-dependent volatile compounds in the pheromonal sources is a new tool to identify the sex-related odours in the animals. Naturally estrus detection by male partner indicates the presence of endocrine-dependent volatiles. The endocrinal dependence of volatile compounds in various pheromonal sources has been reported in mice (Jemiolo et al., 1985; Schwende et al., 1986; Andreolini et al., 1987; Achiraman and Archunan, 2005; Zhang et al., 2007a), in voles (Boyer et al., 1988; Zhang et al., 2007b), in wolves (Raymer et al., 1985) and in cows (Denhard et al., 1991). The present study showed the presence of four endocrine dependent volatiles and nine adult-specific compounds and suggests that testosterone might involve pheromone production in the preputial gland. The three compounds hexadecyl ester
decanoic acid, 9 octadecanoic(Z)-tetradecyl ester and octadecylester octadecanoic acid were seen only in castrated rats and considered as testosterone independent.

In the present investigation, the identified testosterone dependent and the adult specific compounds occurred in the retention time between 25 and 30 min. and are intermediate in cholesterol synthesis. It indicates that cholesterol synthesis in the preputial gland is testosterone dependent. But by visual comparison no change was observed between 5 and 25 min. It is reported that biosynthesis of pheromones is strongly associated with biosynthesis of cholesterol, the essential substrate for steroid hormone synthesis in human axillary apocrine glands (Rothardt and Beier, 2001). There are reports which indicate cholesterol intermediates act as pheromone in rats. For instance, Kannan et al. (1998) reported that the compound famesol, an intermediate in cholesterol synthesis, acts as a pheromone. Similarly, Zhang et al. (2008) reported that the squalene, which is also an intermediate of cholesterol synthesis, acts as a pheromone compound. In the present study, the above reported pheromonal compounds disappeared and decreased respectively after the castration and reconstituted after the testosterone supplementation. Further, among the testosterone-dependent compounds observed in the present study 3,7,11-trimethyl-2,6,10-dodecatrien-1-ol (famesol) alone possesses the molecular weight less than 300 and carbon atoms less than 20. Air-borne pheromones usually contain 5-20 carbon atoms with a molecular weight less than 300 and must be volatile to reach the receiver (Pietras, 1981; Dominic, 1991). Hence, this compound i.e., famesol originated from preputial gland may involve in pheromonal communication.

Thus, the present chapter concludes that the preputial gland showed the functionally significant low molecular mass protein 18 kDa and volatile compounds which varied in response to serum testosterone level, and believed to be involved in pheromone communication. Hence, the forthcoming chapter deals with the identification of this protein.
1.5. **SUMMARY**

1. The preputial gland weight, histoarchitecture, volatile compounds and protein profiles were altered in response to the serum testosterone level.

2. SDS PAGE results revealed the presence of low molecular mass protein i.e., 18 kDa appeared prominently and decreased during castration and reinstated on testosterone treatment.

3. GC-MS results showed 31 compounds in the intact preputial gland, among these nine compounds were adult-specific. Amongst which four compounds namely Geranyl linalool isomer, Oxirane, farnesol and Lanosterol were testosterone-dependent.

4. The three compounds hexadecyl ester decanoic acid, 9 octadecanoic(Z)-tetradecyl ester and octadecylester octadecanoic acid were seen only in castrated rats, considered as testosterone-independent.

5. The results concluded that the testosterone dependent volatiles and the low molecular mass protein i.e., 18 kDa may involve in the pheromone communication.