PHEROMONES IN SOCIAL MOLE-RATS

AND IMPLICATIONS

FOR THE STUDY

OF MAMMALIAN CHEMICAL COMMUNICATION

INAUGURALDISSERTATION

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LIST OF CONTENTS

I ABSTRACT

The work reported in this thesis represents an interdisciplinary approach on chemical (olfactory) communication in Zambian mole-rats of the genus *Fukomys* (family Bathyergidae, Rodentia). Although much attention has been given in this respect to rodents, the role of chemosensation (including acting signals) in these subterranean, eusocial mammals is still not well understood. Individual recognition and signals informing about the reproductive status, both providing the basis of incest avoidance and reproductive skew (Burda 1995), are of special interest particularly because their sensory mechanism remains obscure.

The chapter *Behavioural olfactory bioassays* outlines the evidence for the importance of ano-genital odour in kinship- and individual recognition in mole-rats. Contrary, urine odours did not provide sufficient sensory information in this respect.

Pilot endocrinological and colpocytological examinations in *Fukomys* reveal strong correlation between high mean estradiol- and progesterone values and sexual activity (chapter *Steroids and reproductive status*). Zambian *Fukomys* mole-rats are induced ovulators, primed exclusively through repeated and regular sexual activity and not solely through single copulation or separation from the queen as reported for the Damaraland mole-rats (cf., Molteno & Bennett 2000).

The poorly understood molecular basis (outlined in the chapter *Semiochemicals – HS-SPME-GC-MS-Analysis* and in the chapter *Lipocalines (MUP´s & Aphrodisin) – Proteomics*) of chemosensation was studied, by means of a solvent-free HS-SPME-GS-MS, 2D-PAGE and MALDI-TOF/TOF-tandem MS developed within the framework of this research. Using this approach, it could be demonstrated, that 51 urinary compounds constituting individual volatile urinary pattern of the mole-rat were quite similar even between members of different families. A queen-specific component, 4-nonanone, and similarly 1-hexadecanol, typical for non-reproductive daughters, were identified by mass spectra. Reproductive males (kings) could, however, not be distinguished on the basis of their urinary volatile pattern from non-reproductive males. Males displayed higher amount of ß-caryophyllene in their volatile urinary pattern than females. Although altogether 41 different compounds were identified in urine of families, mole-rats cannot discriminate between different families on the base of odour. Carrot and potato diet influence the urinary composition.

Generally, individual urine variations depend on sex (male or female) and reproductive status (queens or non-reproductive females) and not on individuality per se, supporting the outcomes of behavioural bioassays.

Remarkably, a well-known lipocaline and sex pheromone of the golden hamster (*Mesocricetus auratus*) vaginal discharge, aphrodisin, was identified in the urine of *Fukomys* mole-rats, but without mass polymorphism and in atypically low concentration. In urine of two additionally examined genera of subterranean rodents (*Spalax* spp. and *Spalacopus* sp.), neither aphrodisin nor the lipocaline MUP (major urinary protein), generally present in the house mouse (*Mus musculus musculus* and *Mus musculus domesticus*[1]), was found.

On these backgrounds, it is still questionable, if in Zambian *Fukomys* mole-rats aphrodisin takes on the role of a ligand carrier in the subterranean environment. Ligands such as the prospective 1-hexadecanol or 4-nonanone could be protected from degradation (once urine is deposited). Moreover, the slow release mechanism would prolong the olfactory signal which is proved with MUPs in the house mouse. Still it is not clear, if any or which meaning aphrodisin and/or the urinary volatiles have for the olfactory communication. Further research should concentrate on ano-genital odours (and body odours) because of the clear results of the behavioural assays.

[1] Further abbreviated as *M. m. musculus* and *M. m. domesticus*

II ZUSAMMENFASSUNG

In dieser interdisziplinären Arbeit werden Ergebnisse zur chemischen (olfaktorischen) Kommunikation sambischer Graumulle der Gattung *Fukomys* (Familie: Bathyergidae, Rodentia) vorgestellt. Die Rolle des olfaktorischen Sinneseindrucks (einschließlich wirksamer Geruchssignale) bei diesen subterranen, eusozialen Säugetieren ist trotz vieler Untersuchungen, die bislang bei Nagetieren unternommen wurden, noch weitgehend unverstanden. Das gilt sowohl für die Individualerkennung als auch für die Signale, die über den Reproduktionsstatus informieren. Beide Signale sind Vorraussetzung für die Inzestvermeidung und die Beschränkung der Reproduktion eines Weibchens (der Königin) auf ein bis drei Männchen (Burda 1995), weshalb derartige Signale hier von besonderem Interesse sind.

In dem Kapitel *Behavioural olfactory bioassays* wird die große Bedeutung von Anogenitalgerüchen für die Verwandtschafts- und die individuelle Geruchserkennung bei Graumullen gezeigt. Im Vergleich dazu wurden in diesem Zusammenhang von den Uringerüchen nicht genug Sinnesinformationen geliefert.

Endokrinologische und kolpozytologische Pilotstudien zeigten bei diesen Säugetieren eine starke Korrelation zwischen hohen Östradiol- und Progesteronwerten und der sexuellen Aktivität (Kapitel *Steroids and reproductive status*). Sambische Graumulle sind induzierte Ovulierer. Bei ihnen wird ausschließlich durch wiederholte und regelmäßig erfolgende sexuelle Aktivität die Ovulation angeregt, anders als bei den Damarensis Graumullen, bei denen nur *eine* Kopulation oder die Abwesenheit der Queen ausreicht (vgl. Molteno & Bennett 2000).

Die bislang kaum verstandene molekulare Grundlage der olfaktorischen Kommunikation (vertieft in dem Kapitel *Semiochemicals – HS-SPME-GC-MS-Analysis* und dem Kapitel *Lipocalines (MUP´s & Aphrodisin) – Proteomics*) wurde anhand verschiedener hier entwickelter Methoden, wie der lösemittelfreien HS-SPME-GC-MS, der 2-D PAGE und MALDI-TOF/TOF-Tandem MS untersucht. Hierdurch konnte gezeigt werden, dass sich die individuellen Muster der gefundenen 51 flüchtigen Verbindungen des Urins sehr ähnlich waren. Auch bei dem Vergleich der Mitglieder verschiedlicher Familien ergab sich dieser Befund. Zudem wurde eine queen-spezifische Substanz, 4-Nonanon, sowie 1-Hexadecanol, als typisch für die nicht-reproduktiven Töchter, mit Hilfe von Massenspektren identifiziert. Dagegen konnten reproduktive Männchen (Könige) nicht auf Basis ihrer flüchtigen Urinverbindungen von den nicht-reproduktiven Söhnen unterschieden werden. Männchen unterscheiden sich durch signifikant höhere Mengen an

Caryophyllen im Urin von den Weibchen. Obwohl im Urin der Familien insgesamt 41 verschiedene Substanzen nachgewiesen werden konnten, waren die Graumulle nicht in der Lage, verschiedene Familien am Uringeruch zu unterscheiden. Zudem konnte gezeigt werden, dass sich die Zusammensetzung des Urins jeweils durch eine Karottendiät bzw. durch eine Kartoffeldiät veränderte.

In dieser Arbeit wurde belegt, dass interindividuelle Unterschiede im Urin demnach grundsätzlich vom Geschlecht (Männchen oder Weibchen) und dem Reproduktionsstatus (Königin oder Tochter) abhängen und nicht von der Individualität als solcher, was mit den Ergebnissen der Verhaltensstudien übereinstimmt.

Weiterhin konnte ein bekanntes Lipokalin und Sexualpheromon aus dem Vaginalsekret des Goldhamsters (*Mesocricetus auratus*), Aphrodisin, bei sambischen Graumullen im Urin nachgewisen werden, jedoch in einer auffällig geringen Konzentration und ohne Massenpolymorphismus. Im Urin zweier weiterer untersuchter subterran lebender Nagetiergattungen (*Spalax* spp. und *Spalacopus* sp.) waren dagegen weder Aphrodisin noch das gewöhnlich bei der Hausmaus (*M. m. domesticus*) vorkommende Lipokalin MUP (major urinary protein) im Urin vorhanden.

Vor diesem Hintergrund stellt sich nun die Frage, ob Aphrodisin bei sambischen Graumullen in der subterranen Umwelt die Rolle eines Liganden-Carriers übernimmt. Durch Carrier könnten Liganden, wie potentiell 1-Hexadecanol oder 4-Nonanone, vor Degradation an der Luft geschützt und durch verlangsamte Evaporation könnte, wie bei den MUPs der Hausmäuse, das Geruchssignal verlängert werden. Es ist daher noch unklar, ob und welche Bedeutung Aphrodisin und/oder die flüchtigen Urinverbindungen letztlich für die olfaktorische Kommunikation haben. Weiterführende Untersuchungen sollten sich auf die Analyse von Anogenitalgerüchen (und Körpergerüchen) konzentrieren, da die Verhaltsreaktionen hierbei eindeutig waren.

III GENERAL INTRODUCTION

III.1 Semiochemicals – pheromones

Semiochemicals are single compounds or mixtures of compounds carrying information between organisms in the shared natural environment (Regnier 1971). Different types of semiochemicals are classified (by Nordlund & Lewis 1976) according to their function into two broader classes, pheromones and allelochemicals. The latter mediates interactions between organisms of different species, of which: 1. Allomones adaptively favour the emitting species (the sender), e.g. floral scent attracting pollinating insects (Albone 1984). 2. Kairomones in contrast favour the receiving species, for instance kairomones emitted by roots, bulbs and tubers and detected by a subterranean living herbivore (carrot exudates attracting *Fukomys* mole-rats) (Lange et al. 2005). 3. Synomones favouring both, the sender and the receiver and 4. Apneumones, serving for the chemical detection of dead material. Pheromones, the second large group on the other hand, serve chemical communication between animals of the same species (Wyatt 2003). Figure IIIa below gives an overview about the types of semiochemicals and their function.

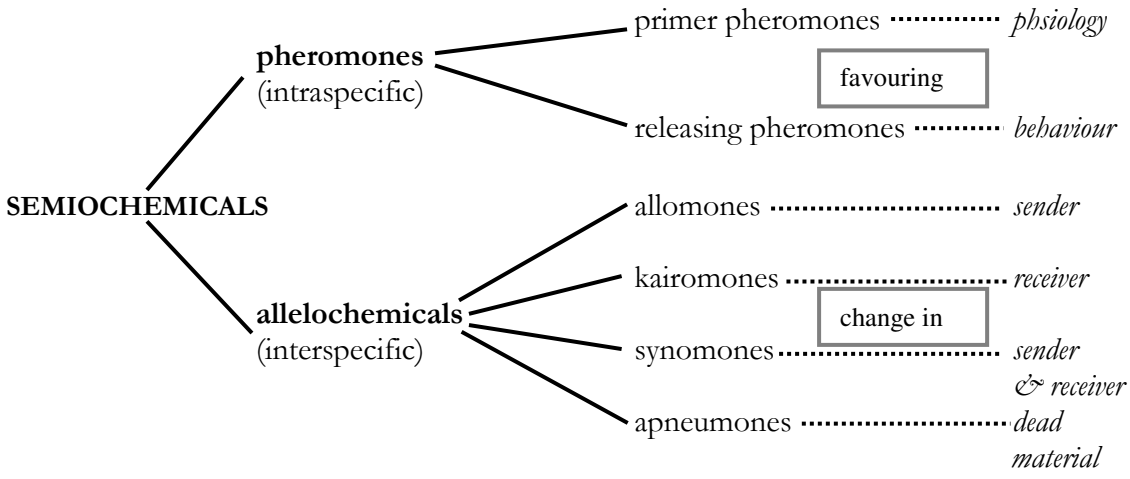


Fig. III.a[2]: Terminology of chemical interaction (adapted from Norlund & Lewis 1976).

The term "pheromone" was originally coined by Karlson and Luscher (1959) as a label for chemosignals that provide information to conspecifics about sex or endocrine status or which release intraspecifically a hard-wired specific reaction. Such specific reaction results either in a specific behaviour (caused by releaser pheromone) or in a

[2] According to Roman numbering of the chapters the figures do not start with I.a

developmental process (caused by primer pheromones). Wilson and Bossert (1963) described releaser pheromones as factors with immediate effects on behaviour of the receiver, whereas primer pheromones as signals, releasing hormones in the receiver with longer term effects. Such endocrinological effects accentuate their relatedness to hormones. The name pheromone originates from "Greek *pherein*, to carry or transfer, and *hormōn*, to excite or stimulate" (Wyatt 2003). Thus in contrast to hormones (acting as internal signals), pheromones act between individuals (releaser and sender, or emitter). Karlson and Butenandt (1959) used the appropriate term ectohormones for pheromones. Those signals were studied by Karlson and Luscher (1959) mainly in insects. However, after more than five decades the study of pheromonal actions has been extended from insects (e.g. Karlson & Butenandt 1959) to a variety of more complex vertebrate species including mammals like mice (e.g. Bruce & Parrott 1960, Novotny et al. 1999a and b), rats (e.g. MacInnes et al. 1986, Lehman-McKeeman et al. 1998), hamsters (e.g. Henzel et al. 1988, Johnston & Rasmussen 1994, Briand et al. 2004) monkeys (e.g. Michael & Keverne 1968) and even humans (Stern & McClintock 1998).

Some authors refer pheromones to a single compound that elicits a specific response (behavioural or physiological, i.e. primer or releaser pheromone). However, in the meanwhile the situation turned to be often more complicated even for insects. Several priming effects in mammals are, however, well described in literature, namely the Witten effect, inducing estrus synchronisation and acceleration when grouped female mice are exposed to male odour, or the Lee-Boot effect inducing estrus suppression in female groupings by absence of male mouse odour (Zidek 1999). Also the Vandenbergh effect (female puberty accelerated by male urine) and the Bruce effect (pregnancy block induced by the presence of a strange male) are well known (Schwende et al. 1985). Other chemosignals induce behavioural effects (releasing pheromones), e.g. intermale aggression or male and female attractiveness (Novotny et al. 1984, Schwende et al. 1985, Novotny et al. 1990).

Particularly in mammals it is difficult, to define what constitutes a specific reaction, since they are subjected to more complex influences (Brennan & Keverne 2004). For instance, eliciting mounting behaviour in male rodents by attractive female sexual signals needs underlying physiological mechanisms (Johnston 2003), thus muddling the dichotomy of the primer and releaser concept. Moreover, responses can vary according to the social context and therefore the current importance, e.g. in the presence of a predator the response of a male to sexual attractant will probably be eliminated (Johnston 2003). This author in addition points out that learning processes are involved in respect to chemical communication. It has been shown most thoroughly for instance, that exposure

of artificial odour during sexual activity of male rats can result in this odour releasing a hormonal response (Graham & Desjardins 1980).

Especially learning processes in the context of signalling individuality and kin recognition, for instance self-reference matching, are of interest here and will be deepened in chapter IV.1.

Many pheromones consist of blends of two or more molecules (Brennan & Keverne 2004), described as a mixture of chemical compounds in relatively precise proportions acting on behavioural, physiological or both levels (Johnston (2000), in: Johnston 2003). Johnson (2000) in addition describes "mosaic signals" to contain a larger number of compounds where the proportions might vary considerably across individuals or classes of individuals. Johnston (2003) accomplishes that such signals are sometimes odours used for individual and kin recognition in mammals.

III.2 Chemical (olfactory) communication and lipocalines

Chemical communication[3] is the oldest form of communication, primarily shared by all organisms including bacteria. This type of signalling developed convergently to a very complex system, so that even the Asian elephant (*Elaphas maximus*) shares its female sex pheromone with some 140 species of moths (Rasmussen et al. 1996). In the course of evolution, leaking hormones developed into pheromones (Sorensen & Stacy 1999, in: Wyatt 2003), alarm pheromones appear to have evolved from compounds released from injured conspecifics or other metabolites into active pheromones (Wyatt 2003). An enormous variety of compounds thus have been evolved. Some pheromones are excreted in urine (e.g. in elephants and mice) whilst others are produced in specialised secretory glands (Wyatt 2003).

Rodents are interesting and intensively studied models for research of chemical communication (Johnston 2003). Rodents are abundant represented by many species and many species are also very abundant (Agosta 1992), being small mammals, they are relatively easy to handle. Most rodent models belong to murids involving mice, rats or hamsters. The house mouse (*M. m. muscuslus* and *M. m. domesticus*) is one of the most investigated species in this respect (Brennen & Keverne 2004). Urine is probably the most important source of odours in this species, affecting a wide range of behavioural and endocrine aspects in conspecifics (Jemiolo et al. 1992, Bennan & Keverne 2004). It contains large quantities of relatively small proteins (major urinary proteins, MUPs) with a

[3] The term chemical communication is preffered here to the term olfactory communication, since it comprises a broader spectrum of chemical signals, apart from odours, including all sorts of semiochemicals particularly pheromones.

molecular mass of less than 20 kDa (kilodalton). MUPs are under testosterone control and are expressed predominantly in adult males (Novotny et al. 1999b) indicating endocrine dependence. Those urinary proteins are members of the lipocalin family (Flower 1996). The lipocalines superfamily belong to a large family of extra cellular proteins, typically around 17-30 kDa in mass (Brennen & Keverne 2004). They are characterized by the ability of binding small hydrophobic molecules. The three-dimensional fold is a remarkable feature which the members of this family share. It forms a hydrophobic binding pocket (cavity binding site) in which they bind small nonpolar molecules (Lücke et al. 1999). Kernel lipocalins share in contrast to outlier lipocalins in addition three short conserved and characteristic sequence motifs, used as a diagnostic of family membership (Flower 1996). To date many further lipocalines are thought to play an important role in chemosignalling.

The urinary proteins have a counterpart on the receiver side of the communication, the odourant binding proteins (OPBs), which are secreted in the nasal olfactory mucosa of vertebrates and bind volatile compounds for perception of odours. Pelosi (2001) distinguished the OBPs, from pheromone binding proteins (PBPs) that are important for perception of pheromones. The latter, which include the mouse MUPs and the rat α2u-globulin, are excreted into urine and are intensively discussed to deliver volatile chemosignals in the environment. Also in the golden hamster (*Mesocricetus auratus*) a 17 kDa lipocaline found in the vaginal fluid of females attracts the attention of males and promotes copulatory activity (Henzel et al. 1988, Mägert et al. 1995, Brennen & Keverne 2004). To date, the role of lipocalines, i.e. ligand-carrier-system, is very controversely discussed. It is not fully understood yet, whether the non-volatile proteins or protein-ligand complexes have a signalling impact (direct model) as favoured by e.g. Mucignat-Caretta et al. 1995, Hurst et al. 2001, Nevison et al. 2003. Novotny's group in contrast favours the indirect model, attributing those proteins a carrier role for protecting small volatile lipophilic molecules (ligands) from degradation, by a slow release mechanism and prolonging the signalling effect (Robertson et al. 1993, Novotny et al. 1999a and b). Novotny and his group identified (1999b) in the house mouse some of the ligands (e.g. 3,4-dehydro-*exo*-brevicomin (DB), 2-*sec*-butyl-4,5-dihydrothiazole (BT) or α- and β-farnesenes), which are active as primer and/or releaser pheromones, e.g. promoting estrus synchronisation or intermale aggression. Lipocalines will be of special concern in chapter IV.4.

Intriguingly any body secretions (urine, faeces, ano-genital excretions, saliva and products of scent glands such as the Harderian gland, parotid gland etc.) are potential routes for pheromonal communication. Johnson (2003) found in the golden hamster discrete

distribution of secretions containing individually distinct information existing at specific locations on the body[4] (cf., fig. IIIb). This means, that in certain odour sources the information about e.g. sex or reproductive status not neccessarily involves information about individuality or other types of information and vice versa, which will be addressed in the chapters IV.1 and IV.3.

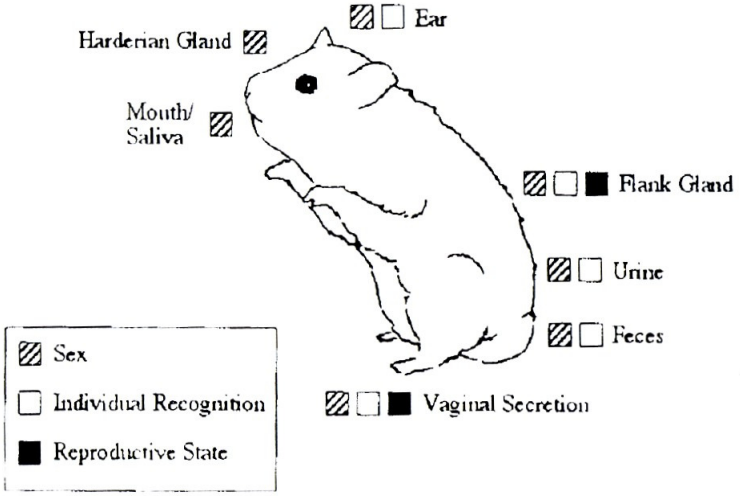


Fig. III.b: Schematic representation of 6 sources of odours on the body of the golden hamster and the distribution of three types of information in those sources (individual identity, sex, and reproductive status (from Johnston 2003)).

The olfactory system of mammals consists of two independent chemosensory systems: the main olfactory epithelium (MOE) sending its signals to the main olfactory bulb (MOB) and the vomeronasal organ (VNO, also known as Jacobson's organ), cf., fig. IIIc. Signals of the VNO are transported to the accessory olfaction bulb (AOB) (Wyatt 2003). Generally, it is still believed that the main olfactory system processes common odours and the (AOB) is specific for pheromones (e.g. Halpern & Martinez-Marcos 2003). Stimulus access to the VNO differs from the MOB by the uptake of non-volatile stimuli, such as lipocalines from urine deposits (or vaginal secretions, scent gland secretions or saliva), which on the other hand does not exclude grabbing also volatile chemosignals (Brennen & Keverne 2004). Xu et al. (2005) in addition demonstrated that a known mouse pheromone,

[4] „It seems likely that most rodents and other mammals have discrete sources of chemical signals or information that differs to some extent. This conclusion is suggested by the mere existence of multiple types of scent glands in many mammalian species and by behavioral observations that suggest that some glands are used or investigated by other individuals in some situations but not others." (Johnston 2003)

2-heptanone, elicits strong signals in both, the MOB and the AOB, thus supporting the latter statement.

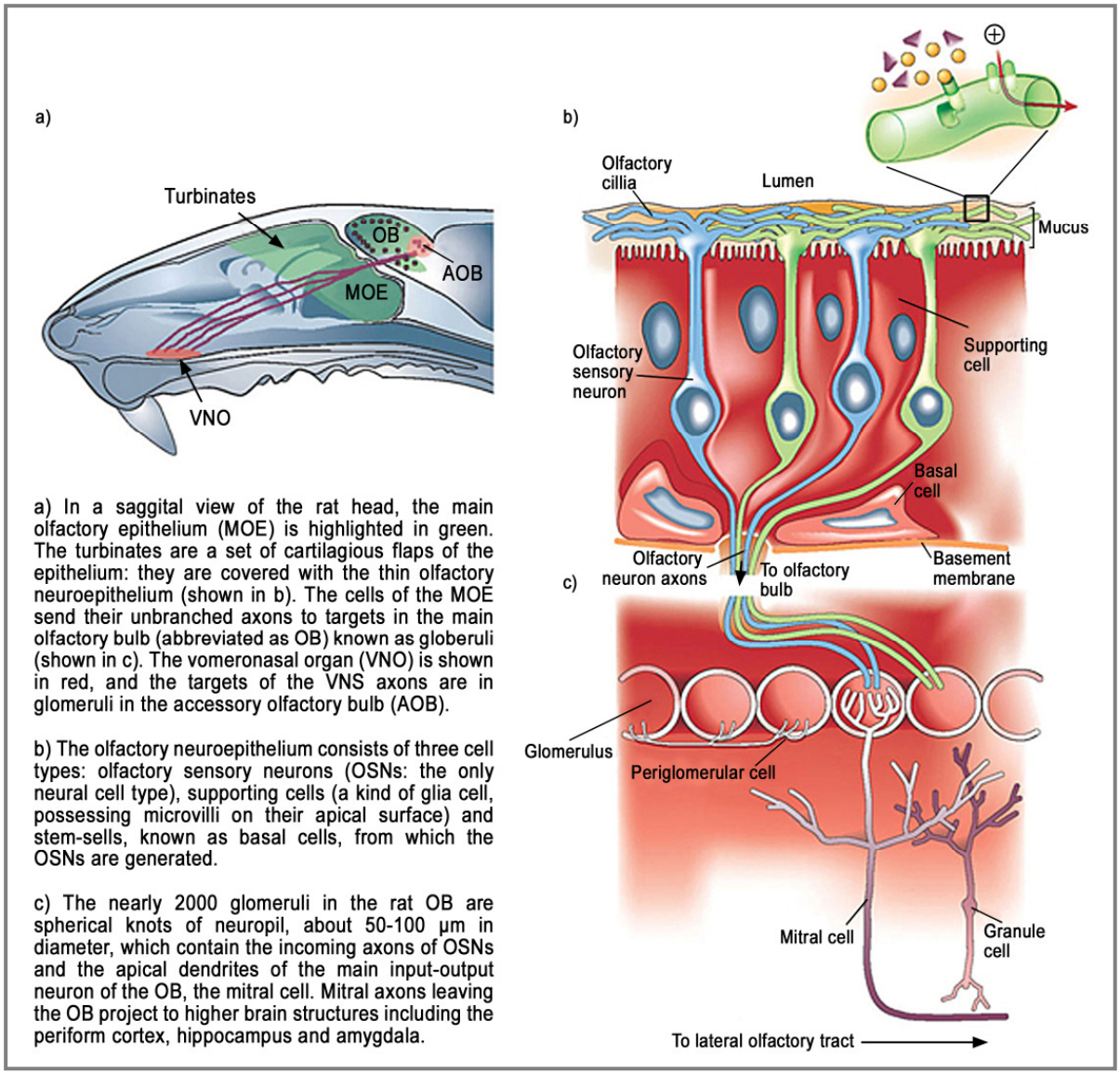


Fig. III.c: Functional anatomy and structure of the early olfactory system. In one of the clearest cases of function following forms in the nervous system, the anatomy and structure of the early olfactory system reflect the strategy for discrimination between a large number of diverse stimuli (adapted from Firestein 2001 and URL2).

III.3 Estrus cycle in the laboratory mouse – a comparative model

The endocrine dependence of mammals on chemical signals is well described, for instance the dependence of MUP on testosterone in males, estrus synchronisation, puberty acceleration elicited by chemosignals (e.g. Novotny et al. 1999b). Moreover, in the female house mouse it is well described, that the urine of estrus females carries a lot of messages to both, males and females (Andreolini et al. 1987), e.g. males are attracted to estrus odours (Davies & Bellamy 1972).

Reproductive processes in female mammals are characterized by cyclic alterations in the female tract and in sexual receptivity. The recurrent period of receptivity or "heat" is called estrus. The estrus cycle has been most extensively studied in laboratory rodents (mice and rats). Small rodents such as voles or the (laboratory) house mouse are examples of spontaneous, polyestrus and continuous breeders according to season, photoperiod and/or laboratory conditions. In the wild during the winter, mice probably postpone the cycle for a period (Conaway 1971). In contrast, for instance the red fox (*Vulpes vulpes*) shows a single estrus in year depending on seasonal environmental conditions. Out of estrus, in anoestrus, the reproductive organs are in a state of quiescence (Mondain-Monval 1977). The estrus cycle involves the whole of the reproductive tract, and it is possible to determine the sexual status of the female mouse by examination of the vaginal smears prepared from the vaginal fluid, namely vaginal cytology (Nelson et al. 1982) or colpocytology.

With the onset of sexual maturity, the ovaries enlarge and start to secrete sex hormones (steroids). Sexual steroids are transported by blood, thus cycling throughout the body: Activation of the ovarian follicles, reflected by cycling steroids, initiates oocyte growth and according to the respective follicle phase, cyclic changes occur. The follicles develop from primordial follicles, making up the "stockpile" of oocytes lasting throughout the live (Lintern-Moore & Pantelouris 1975). In the course of the particular cycle phase, the primordial follicles develop into primary, secondary and the tertiary follicles (also referred to as Graafian follicles) (Hisaw 1947). With this transition the follicle enters the final phase of follicular development followed by ovulation characterized by a burst of the follicle and the release of the oocyte (reviewed in e.g. McGee & Hsueh 2000). Mice are spontaneous ovulators, i.e. no external stimulus is required to initiate ovulation. Diurnal rhythms of light and dark however influence the time of surge of the luteinizing hormone (LH) (Miller et al. 2004).

The mouse estrus cycle is composed of four stages, beginning with the proestrus, followed by estrus (where typically ovulation takes place), metestrus and diestrus

(Allen 1922). Together with interstages up to seven stages are differentiated (Nelson et al. 1982): The proestrus is characterized by high estrogen concentration stimulating the cell division in the uterus and vagina. When estrogen reaches its peak during this stage, it triggers a surge of LH that initiates the ovulation. In addition, maximal concentration of FSH is also observed during proestrus (Murr et al. 1973). The proestrus is characterized by nucleated epithelial cells (reflecting the effects of elevated estrogen levels on these tissues).

During the estrus, in mice after LH-surge (Bingel & Schwartz 1969), the estrogen level is elevated in the late diestrus and early estrus and ovulation occurs (Traurig 1970, Walmer et al. 1992). The female is receptive to male during this stage, a behavioural consequence of elevated estrogen concentrations during this stage (cf., fig. IIId) (Gidley-Baird et al. 1986, reviewed in Wang & Dey 2006). The epithelium of the vagina having reached its maximal thickness, begins to slough off cornified cells.

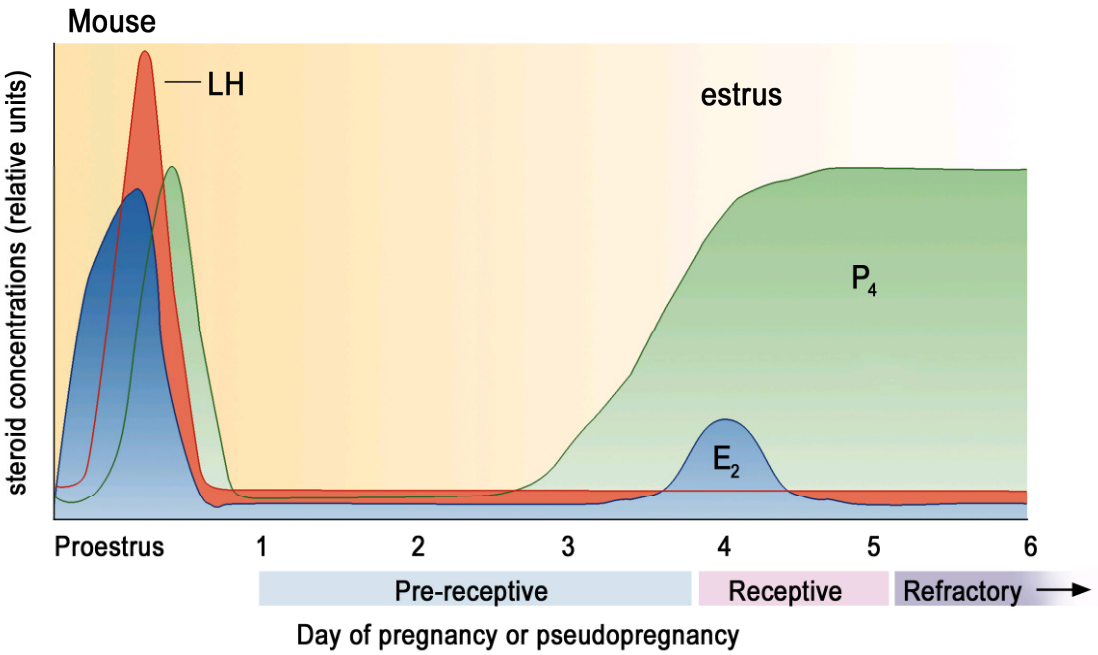


Fig. III.d: The window of uterine receptivity and estrus cycle in mice: The major hormones that specify uterine receptivity are the ovarian steroids. Progesterone (P_4) and estrogen (E_2). In mice, the estrus cycle is short (~4 days) and often irregular. Therefore, it is difficult to determine the receptive phase during the cycle. Uterine sensivity to implantation is classified into pre-receptive, receptive and non-receptive (refractory) phase. During the pre-receptive phase, the uterus is unable to initiate implantation, but the uterine environment is less hostile to blastocyst survival. In contrast, during the refractory phase, the uterine environment is unfavourable to blastocyst survival. In mice, the uterus is receptive on day 4 (estrus), whereas it is pre-receptive on days 1-3, and by the afternoon on day 5 it becomes non-receptive (refractory) to implantation (adapted from Wang & Dey 2006 and URL3).

The metestrus coincides with declining concentration of estrogen following the LH surge and ovulation. Corpora lutea have formed and atresia is widespread among remaining

follicles (atretic follicles). As estrogen concentration declines, growth in the mucosa ceases and the epithelium shows signs of degeneration. In the vagina sheets of epithelial cells slow off and many leucocytes are present (Nelson et al 1982).

The diestrus is the quiescent phase of the cycle. Estrogen concentrations are still low and in the absence of mating, the corpora lutea produce little progesterone (Greenwald & Rothchild 1968, Humphreys et al. 1985). The vaginal smear reveals epithelial cells and leucocytes.

In sum, leucocytes represent the predominant cell population during the early proestrus, the late metestrus and during diestrus. The late proestrus and the estrus are solely dominated by cornified cells, whilst both leukocytes and cornified cells are absent (Nelson et al. 1982).

Endocrine regulation of breeding is well studied in South African mole-rats (*Fukomys damarensis* and several *Cryptomys* species) or naked mole-rats, *Heterocephalus glaber* (reviewed in Bennett et al. 2007) but up to now, comparative data on other species of African mole-rats including *Fukomys* are absent. Thus far, neither hormonal assays nor colpocytological research, as a marker for ovulation, have been reported for other eusocial *Fukomys* mole-rat species, but *F. damarensis*. The endocrine dependence of some chemical signals led me to find out more about the reproduction in *Fukomys* mole-rats and probable correlations to chemical signals, outlined in respective chapters of this thesis.

III.4 Subterranean eusocial *Fukomys* mole-rats

Ansell's and Kafue mole-rats, *Fukomys anselli* and *Fukomys kafuensis*[5] (formerly designated as *Cryptomys*, cf., Kock et al. 2006) called here as *Fukomys* mole-rats, are subterranean rodents (family Bathyergidae) from Zambia. The family Bathyergidae includes six genera: *Bathyergus, Fukomys*, *Cryptomys*, *Heliophobius, Georychus,* and *Heterocephalus* (Faulkes et al. 2004, Ingram et al. 2004, Kock et al. 2006, van Daele et al. 2004).

Fukomys mole-rats live in large, multigenerational families founded by a single pair monopolizing the breeding. The offspring remain in the family as helpers to their parents and younger siblings and do not breed. Compared to helpers known in monogamous mammals or birds (e.g. Schoech et al. 1996), the "workers" in mole-rats show long lasting philopatry encompassing overlap of several generations (= litters) of siblings occurring in the nest in parallel. Although the "workers" in *Fukomys* do not reproduce, they are physiologically fertile. This social structure resembles that of social insects or naked mole-rats and can be denoted eusociality (cf., Burda et al. 2000 and literature therein).

[5] *Fukomys anselli* and *Fukomys kafuensis* are further abbreviated: *F. anselli* and *F. kafuensis*.

Accordingly, the breeding female (mother of the family) uses to be denoted as a queen, and her non-breeding offspring constitute the workforce. Under laboratory conditions, a new family can be founded only by pairing two unfamiliar animals. The death or removal of a breeder results in reproductive quiescence of the family, showing that the mechanism constraining breeding within a family is strong incest avoidance (Burda 1995). Female workers do not breed because they do not copulate. They do not mate simply because the male family members (brothers and fathers) are sexually unattractive for them and vice versa. This incest avoidance is based on individual recognition and social memory, and not on semiochemical or behavioural suppression (Burda 1995).

III.5 Ambition for the thesis

The present thesis deals with different aspects of chemical (olfactory) communication (mainly of urine odours) in Zambian *Fukomys* mole-rats on four different interdisciplinary research levels: behavioural, endocrinological, chemo-analytical (volatile urinary components) and proteomical (non-volatile urinary components).

The behavioural bioassays were performed in order to enlighten the most informative value of two odour sources for individual and kin recognition as much as odour attractiveness of females of different sexual activity respectively.

Endocrinological and estrus cycle investigations should contribute to deeper understanding of the reproductive skew and its impact on differences in the metabolism of sex steroids and chemosignals between males and females of different reproductive status.

To interrelate hitherto behavioural assays, chemo-analytical and proteomic research were designed to identify urinary volatile compounds (potential ligands) and their prospective lipocaline carriers.

IV CHEMICAL (OLFACTORY) COMMUNICATION

IV.1 BEHAVIOURAL OLFACTORY BIOASSAYS

IV.1.1 INTRODUCTION

Mammals of many species communicate by means of odourous signals, thus obtaining information about the senders' individuality (e.g. Brown 1987, Hurst et al. 2001), gender (Schwanz & Lacey 2003), dominance status (Novotny et al. 1990, Clarke & Faulkes 1999) group membership (Mateo 2003), kin relatedness (Clarke & Faulkes 1999, Todrank et al. 1999, Zenuto & Fanjul 2002), and affiliation with a particular species (Heth & Todrank 2000, Heth et al. 2001, Munclinger & Frynta 2000).

The use of olfactory cues that allow discrimination between individuals has been widely reported in rodent species such as *Rattus norvegicus* (Brown et al. 1987), *Mus domesticus* (Novotny et al. 1999a and b, Hurst et al. 2001), *Mesocricetus auratus* (Johnston & Rasmussen 1984, Tang-Martinez et al. 1993) and *Phodopus campbelli* (Lai & Johnston 1994). Similar studies on subterranean species include *Spalax ehrenbergi* (Nevo et al. 1987, Shanas & Terkel 1996), *Fukomys* spp. (Heth et al. 2002) *Ctenomys* sp. (Zenuto & Fanjul 2002, Fanjul et al. 2003) and *Spalacopus cyanus* (Hagemeyer & Begall 2006).

Besides many functions of further chemical communication (described above), olfactory communication is expected to be especially complex in social mammals since the maintenance of appropriate social structure, e.g. restriction of reproduction to one single breeding pair while the offspring remain non-reproductive, largely depends on the ability of group or family members to distinguish between one another and subsequent differential response (Fanjul et al. 2003). Since *Fukomys* mole-rats are xenophobic towards unfamiliar conspecifics the response, i.e. aggressiveness or amicability, might be influenced by previous encounters (Zenuto & Fanjul 2002).

Cumulative evidence indicates that individuals of varying degrees of genetic relatedness exhibit graded similarities in the qualities of their odours (i.e. the closer the genetic relatedness, the more similar the odours). This relationship has been referred to as odour-genes covariance (Heth & Todrank 2000). The discrimination mechanism that enables this relationship to be detected has been termed "genetic relatedness assessment through individual odour similarities" or "G-ratios" (Heth et al. 2003, Todrank & Heth 2003). When a subject encounters another individual it can assess the genetic relatedness

between them by comparing the degree of the similarity between the individuals' odour and its own odour – a graded self-referencing process (Todrank & Heth 2003). This contrasts with phenotype-matching mechanism in which an individual learns the olfactory characteristics (odour templates) of individuals (e.g. by direct familiarisation with nest-mates) and matches the template of an encountered (novel) individual against a composite of previously acquired templates (Halpin 1986). This mechanism also includes the possibility of learning about one's own phenotypic characteristics and using them as a reference (Dawkins 1982, Waldmann et al. 1988, Mateo & Johnston 2000).

Heth et al. (2002) reported the existence of ano-genital kinship odours within families of giant mole-rats (*Fukomys mechowii*), demonstrating that closely related individuals have similar odour qualities. The authors applied the habituation-generalization technique. In fact, giant mole-rats recognize their kin still after almost 5 month of separation (Bappert & Burda 2005). On the other hand, the smaller Ansell's mole-rats (*F. anselli*) forget their siblings "already" after about three weeks of separation (Burda 1995), yet the recognition mechanisms; which play a major rule in this species; remain unresolved and the habituation approach has not been applied in the study of individual and kin-recognition in this species so far.

In Ansell's mole-rats, families consist of the reproductive pair and their offspring and mating between relatives (parent – offspring or brother – sister) does not occur. Individual recognition (probably mediated by olfaction) may help to establish and maintain this incest taboo.

Burda (1995) found that familiar individuals are not attractive as mating partners. Although, any unfamiliar animal of an opposite sex may arouse sexual interest, there still seem to be differential preferences for a certain male or female. Therefore, it would be of interest to examine which factors, apart from unfamiliarity, affect the attractiveness of the individuals' odour. Assumed that the female odour is influenced by the reproductive status and that this status furthermore is influenced by primed circulating hormones (see chapter IV.2), the metabolites which may be olfactory active, two different mechanisms of partner choice may be expected: firstly by individual recognition and secondly by recognition of reproductive active signals. Accordingly, two different types of experimental set-ups were performed.

The first method that enables assessment of both odour discrimination and odour similarity is the habituation-generalization-test (Todrank et al. 1998). This test assesses the animals' ability to discriminate between odours in general, and to detect odour similarities of more closely related individuals compared with those of a less closely related individual. Repeated exposure to the odour of one individual results in reduced interest in this odour

– a process that is referred to as habituation (cf., Halpin 1986). During the first test-trial, which follows the habituation, a second odour from a donor that is closely related to the habituation odour donor is presented. The odour offered during the second test-trial (presented either consecutively or simultaneously with the first test odour) originates usually from a genetically less similar donor. If there is a biologically significant difference to the reference odour, the tested individual reveals renewed interest in odour investigation. To sum up, this test is appropriate to detect the subjects' ability to discriminate between odours of different individuals or to detect similarities between them. However, conclusions about the subjects' preference or recognition as a cognitive process cannot be drawn (cf., Halpin 1986).

Previous odour-genes covariance tests have demonstrated that odours of close relatives are very similar; thus, subjects may not be able to distinguish between the siblings' odours. If these are too similar, the sniffing time in test one may not increase significantly in comparison to the third habituation trial. In contrast, if individuals distinguish between siblings, the sniffing time in test one should increase significantly.

The second method to study the odour preference is a preference test in which two odours of opposite sex are presented simultaneously. Both test designs were conducted with ano-genital and urine odour to find the odour source most relevant for individual- and kin recognition. The preference test was furthermore conducted with a mixed odour, prepared by mixing urine with ano-genital odour, to test the potential amplification effect of both signals.

Family members of *F. anselli* and *F. kafuensis* greet each other by nose to nose sniffing, cheek rubbing and, above all, by very intensive ano-genital sniffing. Previous observations and mating experiments showed that after nose to nose sniffing, mole-rats tend to sniff their flanks and finally intensively the ano-genital area, whilst females frequently demonstratively present their ano-genital area to a male by lifting their backside (=lordosis) repeatedly to the nose of the strange male. Furthermore, I observed repeatedly demonstrative urine depositing by lifting the backside against the wall of the cage suggesting marking function of this behaviour and signalling impact of this odour source. Urine as a source of odour is relatively easy to collect and handle.

Many studies on several rodent species, such as *Spalax* spp. and especially on laboratory- and the wild house mouse (*M. m. musculus* and *M. m. domesticus*), tested urinary odours (e.g. Jemiolo et al. 1992, Cavaggioni & Mucignat-Caretta 2000, Nevo et al. 1987, Heth et al. 1996a and b). Heth et al. 2002, however, argued that ano-genital odour in *Fukomys* mole-rats is of high relevance (see above). Nevertheless, urine odour has never

been tested in *Fukomys* mole-rats so far. Moreover to date, no systematical study has been conducted to compare different sources of odours with each other.

This part of my thesis therefore should fundamentally contribute 1) to determine the significance of three different kinds of odours for this genus by comparing them with each other on behavioural level, 2) to explore the extent to which olfaction of those different odours could be useful for recognition in a highly social species, and 3) to a more detailed understanding of attractiveness of odour of a reproductive female (queen), compared to their non-reproductive daughters to an unfamiliar male.

IV.1.2 MATERIAL AND METHODS

IV.1.2.1 Animals

The *Fukomys* mole-rats (*F. anselli, F. kafuensis*, and their hybrids) from eight families were tested under two different conditions, referred to as "habituation-generalization test" and "preference test" (see above). The animals were either born in captivity or caught in the wild. They are housed in glass terrariums of varying size (depending on the size of the family), filled with a thick layer of horticultural peat as ground substrate. For further details on housing conditions, see Burda (1989, 1990). Animals were fed with the same diet of fresh carrots, potatoes, and apples supplemented with lettuce and cereals. Mole-rats do not drink free water. The animal room at the Department of General Zoology, University of Duisburg-Essen, campus Essen, had a natural dark-light cycle with room temperature ranging between 20° C and 25° C.

To avoid confounds and to guarantee comparable motivation levels of the test-animals, in the habituation setting, subjects were presented odours of individuals of the same sex because the oestrus-cycle of this species was not known at the time of the study (see chapter IV.2). Most animals took part in both, habituation and preference tests, but with almost one year separation between the two test conditions. In order to increase the number of test-animals, occasionally a subject was involved up to twice in one test procedure with not less than two weeks pause between. This was the case in the familiar condition (see below) as this design demands testing of at least four same-sex-individuals of one family.

Ethical note

All efforts were taken to minimize stress of the animals. Animal husbandry and all the experimental procedures complied with German regulations on the care and use of experimental animals.

Habituation-generalization test

Tests were conducted during early morning, between 8 a.m. and 12 a.m. in the spring and summer (may until september), 2003. Odour donors used during habituation and test-trial one (see below) were full siblings. Subjects of a respective family had never been in contact with any member of the other two families, thus, they were strangers to them.

Altogether 20 adult female and 18 adult male *Fukomys* mole-rats from eight different families were used as test-animals. As the number of available adequate animals for this test-procedure was limited, eight females and five males of the test-animals (=subjects) served simultaneously as odour donors.

Preference test

The study was conducted from August 2004 until February 2005, from 7 a.m. to 10 a.m. Altogether 29 males of fifteen different families were tested. Nine reproductive males (kings = rK) of *F. anselli* were used as subjects in three different odour tests. First they were tested with ano-genital odour, second with fresh urine odour and then with the mixed odour of urine and ano-genital odour respectively. Furthermore, twelve non-reproductive males (sons = nrm) from *F. anselli* and eight non-reproductive hybrid males served as test-animals. All those animals originated from families consisting of the king (K), the queen (rQ) and at least one son (nrm) or daughter (nrf). Each subject was at least one year old (except of two nrm which were ten month at the first odour test).

Five different queens and four different nrf of *F. anselli* served as odour donors. The use of two nrf-hybrids as odour donors was restricted to hybrid test-animals.

IV.1.2.2 Odour collection

Urine was collected by picking up the animal gently on its backside skin fold out of the cage and letting it urinate directly into a 1.5 ml Eppendorf-tube. If the animal did not urinate immediately, it was put into a clean bucket, and usually urinated within a short time. The urine was collected with an Eppendorf pipette into the Eppendorf-tube, centrifuged at 8,000 rpm for eight seconds and the supernatant was pipetted into a fresh Eppendorf-tube to avoid contamination with dirt from the animals' pelage. The odour donors were returned back to their respective home cages. For tests, 150 µl of freshly collected urine were pipetted onto a clean glass plate and smeared out evenly with a clean microscope slide. In order to ensure that no additional odours contaminated the plates, disposable rubber gloves were used that were changed before touching another animal.

The ano-genital smell was transferred to a clean glass plate by picking up the odour donor and gently rubbing its ano-genital region five times along each side of the glass plate and an additional two times diagonally (shown in the fig. VII: A1, appendix).

IV.1.2.3 Habituation-generalization test

Test chamber

The experiments were performed outside the animal room to avoid odourous or acoustical disturbances during the test procedure. A clean glass terrarium (21 x 40 x 25 cm) served as the test chamber. Odours were presented on glass plates (9 x 11 x 0.5 cm) that were positioned at one side of the terrarium. The glass plates were fixed to the floor of the test chamber with double-sided adhesive tape. During each trial, the test chamber was covered with a perspex plate (36 x 46 x 0.8 cm). After each test procedure the test chamber and the cover plate were thoroughly washed with water, additionally cleaned with 3% acetic acid and water once more, and finally dried with paper tissue. Terrarium outer walls were covered with paper, except for the wall in which the video camera was placed to avoid the disturbance of the video recording by the confusing background (note that mole-rats themselves do not perceive images and are thus virtually blind; fig. IV.1.1).

Fig. IV.1.1: Test-chamber with a sniffing mole-rat at odour plate during the habituation-generalization test.

Test procedure

Prior to each test procedure, the subject was given three minutes (min) to acclimate to the test chamber. Each test was video-recorded using the NOLDUS® video analysing system.

Following the habituation-generalization test procedure by Heth et al. (2002), two experimental conditions (familiar and strangers) were used. Ano-genital odours or 150 µl of freshly collected urine odours were presented to a subject on five consecutive trials. The first three trials were habituation trials (H 1-3), in which the odour came from the same donor. The fourth and fifth trial were test-trials (T 1-2), in which the odours from two other individuals were presented. Each trial lasted three min with a one min inter-trial interval during which freshly prepared odour was transferred to a clean glass plate. Generally, the three min trial started when the subject had its first contact with the odour plate.

The sniffing times (in seconds) at the stimulus source were recorded by means of mechanical stop watches. The video-tapes were used as control. The sniffing was defined as a close (about 0.5 cm) contact of the animals' nose to the stimulus area of the glass-plate. To exclude that the glass plate per se raised the interest of mole-rats, six individuals (three males and three females) were subjected to control-test (instead of urine or ano-genital odour, 150 µl water was smeared out on the glass plate).

In the **familiar sibling condition** the subject was exposed to the odour of a same-sex familiar sibling from its own family for three consecutive habituation trials (labelled throughout the text as "sibling one"). During the first test-trial the subject was confronted with the odour of a different familiar same-sex sibling from its own family (labelled as "second sibling") and during the second test-trial to the odour of an unfamiliar individual from a different family was presented (labelled as "stranger").

In the **strangers' condition**, the subject habituated to the odour of an unfamiliar individual from a different family (i.e. a stranger to the subject) in consecutive trials (labelled as "stranger one"). During the first test-trial the odour of a sibling of the first odour donor was presented (labelled as "stranger two"). During the second test-trial the subject was tested with the odour of another unfamiliar individual but from a different family than the first donor (labelled as "novel stranger"). The relationships between individuals of both test conditions and the code used here are given in table IV.1.1, next page.

Tab. IV.1.1: Relationship and the **code** between test-subjects and all odour donors in the familiar condition and the strangers' condition. The unfilled, chequered, and grey circles are individuals from family A, B and C. H = habituation phase, T = test-trial, fam. = family.

	subject	**odour donor H 1-3**	**odour donor T 1**	**odour donor T 2**
familiar condition	indiv. 1 (fam. A) 	indiv.2 (fam. A) 	indiv. 3 (fam. A) 	indiv. 1 (fam. B) 
code		"sibling one"	"second sibling"	"stranger"
strangers' condition	indiv. 1 (fam. A) 	indiv. 1 (fam. B) 	indiv. 2 (fam. B) 	indiv. 1 (fam. C) 
code		"stranger one"	"stranger two"	"novel stranger"

IV.1.2.4 Preference test

Test chamber

A clean glass terrarium (21 x 40 x 25 cm) served as the test chamber. Odours were presented on clean glass plates (9 x 11 x 0.5 cm) that were positioned at one side of the terrarium. These glass plates were divided by a marked line at the bottom, and two odours were presented simultaneously. A clean glass plate of the same size was placed in the cage opposite to the odour plate as a control. In contrast to the habituation-generalization test, using a control plate during the test-session was possible, since the manual handling in this test-procedure was easier. Both glass plates were fixed to the floor of the test chamber with double-sided adhesive tape. During the experiment the test chamber was covered with a perspex plate (36 x 46 x 0,8 cm), fig. IV.1.2 next page.

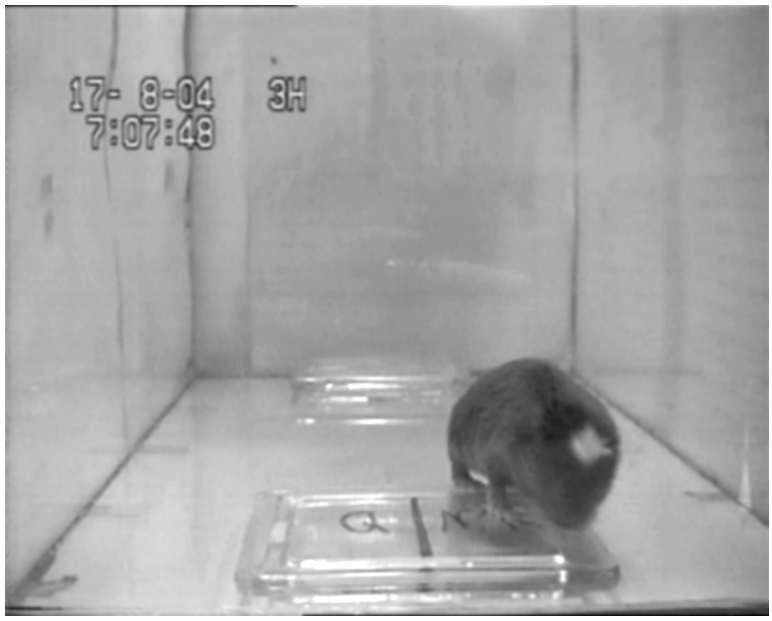


Fig. IV.1.2: Test-chamber with sniffing mole-rat at odour plate during the preference test.

Test procedure

To get the animal accustomed to the test chamber each subject was transferred into it for five min before test procedure. Each test was video-recorded using the NOLDUS® video analysing system. The mixed odour of urine and ano-genital odour was prepared by pipetting 50 µl freshly collected urine onto the ano-genital area of the same test-animal and subsequent transferring to the glass plate in the same manner as described above for ano-genital odours. Otherwise the same caution to avoid artefacts and odour mixing was taken as in the habituation-generalization test. To avoid odour mixing along the parting line of the glass plate, the mole-rats ano-genital area was mainly gently rubbed at the angle and the first third of the right and left side of the glass plate respectively.

Right after the five min acclimatisation time, both the odourized glass plate, prepared with the odours of a strange queen and her daughter simultaneously and the control glass plate were positioned at opposite sides of the terrarium. Sniffing times were recorded in the same manner as in the habituation-generalization test.

IV.1.2.5 Statistical analysis

Throughout the text, all mean sniffing times are given as $\bar{x} \pm$ SD (standard deviation). For the **habituation-generalization test**, the Mann-Whitney *U*-test for independent samples was used to compare the mean times males and females spent sniffing at a stimulus. Due to the different sniffing times in test-trial two with urine as odour source in the familiar and stranger condition, the Mann-Whitney *U*-test was processed consecutively. To test for

decrease in time during consecutive habituation trials the Friedman's rank test was used. Significant differences of pair wise data were tested by the Wilcoxon-rank-test for paired samples (i.e. mean sniffing times in test one versus test two at odourized plate). All statistical analyses were performed with SPSS (version 11).

In the **preference test**, the data were normally distributed (Kolmogorov Smirnov-test) but the variances were partly not homogeneous (Levene-test). Consequently, non-parametrical statistical tests were applied. To test the preference for one of the three stimuli (simultaneously rQ, nrf, and control), mean sniffing times were compared by the Kruskal Wallis ANOVA (*analysis of variance*) for more than two independent samples. The post-hoc-test (Scheffe) finally showed the tendencies of significance. Furthermore, differences in the mean time of sniffing at the odour sources (ano-genital odour, urine odour and the mixed odour of urine and ano-genital odour) were tested with Kruskal Wallis ANOVA and post-hoc to find the significances, since also here the data were partly not homogeneous in variance. Mann-Whitney *U*-test for independent samples was performed to compare mean times between nrm and K at the stimulus.

IV.1.3 RESULTS

IV.1.3.1 Habituation-generalization test

After they prodded more or less accidentally with their noses at the odourized glass plate, the mole-rats sniffed intensively at it. Intensive head movements, head bobbing, which are discussed to be typical for caviomorph rodents (cf., Hagemeyer et al. 2006) were not observed in mole-rats. Nevertheless, after their interest for the stimulus had been aroused, they fixed their noses to the odour source and bit into the plate (cf., fig. IV.1.2).

Ano-genital odour

During the first odour contact (sniffing time during habituation 1) males and females showed similar behaviour in the test chamber with comparable sniffing times at the odour plate in the familiar condition (males: 11.3 ± 4.5 s, females: 8.5 ± 5.5 s) as well as in the strangers' condition (males: 9.2 ± 3.3 s, females: 12.9 ± 7.9 s; tab. IV.1.2).

All subjects habituated to the odour of a familiar sibling (sibling one) as shown by significant decrease in time the animals spent sniffing in three consecutive trials (Friedman's rank-test: females: $\chi^2 = 14.600$, $p = 0.001$, $df = 2$, $n = 10$; males: $\chi^2 = 10.889$, $p = 0.004$, $df = 2$, $n = 9$; cf., tab. IV.1.2).

However, mean times a subject spent investigating the odourized plate during the first test-trial (i.e. the ano-genital odour of the second sibling) were highly increased in comparison to the third habituation trial (i.e. sibling one). The difference is highly

significant (Wilcoxon-rank-test: Z = -3.179, p = 0.001; fig. IV.1.3, tab. IV.1.3). Significant increase holds true also for separated data of males and females.

During the second test-trial the subject was confronted with the ano-genital odour of the stranger and the sniffing time again was significantly higher than in the first test-trial (Wilcoxon-rank-test: Z = -3.30, p = 0.001) (fig. IV.1.3, tab. IV.1.3). Interestingly, males sniffed significantly longer at that stimulus than females (Mann-Whitney *U*-test: males: 22.0 ± 11.0, females 9.7 ± 3.9, p = 0.006, U = 12.0; fig. IV.1.4, tab. IV.1.4).

The control test, however revealed minor interest of subjects on glass plates "odourized" with water, since neither habituation occurred (Friedman's rank-test: $\chi^2 = 3.2$, p = 0.311, n = 6, df = 2) nor an increase in sniffing at the plate in the course of test one and test two occurred (Wilcoxon-rank-test: H3-T1, p = 0.249, Z = -1.153; T1-T2, p = 0.753, Z = -0.314, T1-T3, p = 0.463, Z = -0.734; tab. IV.1.3).

Tab. IV.1.2: Friedman-test for sniffing times (s = seconds) in three consecutive trials at the odour of sibling one of the test-animal in familiar condition and at the odour of stranger one during habituation-phase 1 until habituation-phase 3. Significance level $p < 0.05$, H = habituation phase.

test	H1 [s]	H2 [s]	H3 [s]	n	χ^2	p	df
ano-genital familiar	9.8 ± 5.2	5.4 ± 2.7	4.6 ± 3.4	19	24.947	0.000	2
ano-genital familiar ♂	11.3 ± 4.5	5.8 ± 2.1	5.5 ± 3.2	9	10.889	0.004	2
ano-genital familiar ♀	8.5 ± 5.5	5.0 ± 3.2	3.7 ± 3.5	10	14.600	0.001	2
ano-genital stranger	11.8 ± 7.0	7.2 ± 5.6	5.4 ± 4.5	20	22.900	0.000	2
ano-genital stranger ♂	9.2 ± 3.3	5.5 ± 2.5	3.4 ± 1.2	6	7.000	0.030	2
ano-genital stranger ♀	12.9 ± 7.9	8.0 ± 6.4	6.2 ± 5.1	14	16.000	0.000	2
urine familiar	12.2 ± 5.0	9.9 ± 4.5	5.5 ± 3.9	16	21.500	0.000	2
urine familiar ♂	14.6 ± 6.0	11.9 ± 5.1	5.0 ± 2.0	6	10.333	0.006	2
urine familiar ♀	10.8 ± 3.9	8.7 ± 3.9	5.8 ± 4.8	10	11.400	0.003	4
urine stranger	9.5 ± 5.5	5.3 ± 4.7	3.5 ± 2.5	19	24.827	0.000	2
urine stranger ♂	7.4 ± 4.4	4.5 ± 2.9	3.7 ± 3.3	5	7.600	0.022	2
urine stranger ♀	10.2 ± 5.8	5.6 ± 5.2	3.4 ± 2.3	14	17.491	0.000	2
control	11.1 ± 4.4	8.9 ± 5.8	7.2 ± 4.6	6	2.333	0.311	2

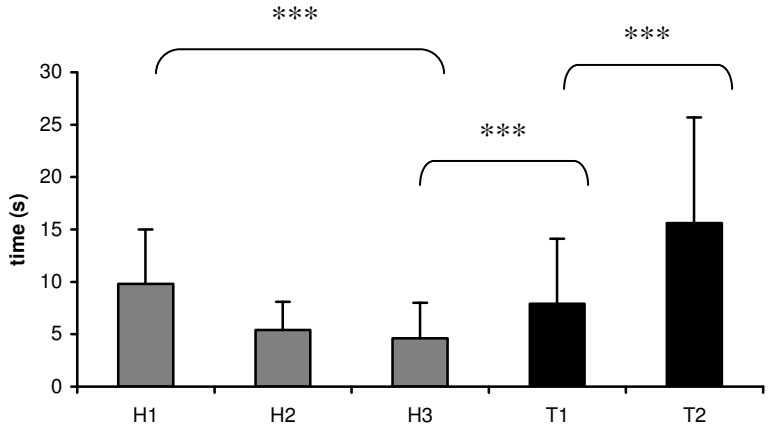


Fig. IV.1.3: Mean (± SD) times that subjects spent investigating **ano-genital odours** in a habituation-generalization test (**familiar condition**). The first three grey columns represent three consecutive habituation trials (H1-H3, during which the subject was presented the ano-genital odour of sibling one) followed by two consecutive test-trials. During test-trial one = T1 the odour of a second sibling was presented to the subject; the odour in test-trial two = T2 came from the stranger. Significance level $p < 0.05$, *** = $p < 0.001$.

Tab. IV.1.3: Wilcoxon-rank-test of differences in sniffing times between H3 and T1, T1 and T2, H3 and T2. H = habituation by sniffing at the odour of sibling one in familiar condition (strangers' condition = odour of stranger one), T1 = sniffing times at the odour of the second sibling in familiar condition (strangers' condition = odour of stranger one), T2 = sniffing times at the odour of the stranger in familiar condition and stranger two in strangers' condition. Significance level $p < 0.05$, s = seconds.

test	$\bar{x} \pm$ SD [s]			n	paired test p Z		
	H3	T1	T2		H3-T1	T1-T2	H3-T2
ano-genital familiar	4.6 ± 3.4	7.9 ± 6.2	15.6 ± 10.1	19	0.001 -3.179	0.001 -3.300	0.000 -3.823
ano-genital familiar ♂	5.5 ± 3.2	10.0 ± 7.4	22.0 ± 11.1	9	0.021 -2.310	0.011 -2.547	0.008 -2.666
ano-genital familiar ♀	3.7 ± 3.5	5.9 ± 4.5	9.7 ± 3.9	10	0.022 -2.295	0.028 -2.191	0.005 -2.805
ano-genital stranger	5.4 ± 4.5	6.3 ± 3.9	14.9 ± 10.3	20	**0.173** -1.363	0.001 -3.472	0.000 -3.763
ano-genital stranger ♂	3.4 ± 1.2	4.9 ± 2.4	13.5 ± 9.9	6	**0.340** -0.954	0.046 -1.992	0.043 -2.023
ano-genital stranger ♀	6.2 ± 5.1	6.9 ± 4.4	15.5 ± 10.8	14	**0.258** -1.131	0.004 -2.856	0.001 -3.202
urine familiar	5.5 ± 3.9	10.7 ± 5.8	13.6 ± 12.8	16	0.001 -3.408	**0.605** -.517	0.007 -2.715
urine familiar ♂	5.0 ± 2.0	10.7 ± 5.7	20.1 ± 18.6	6	0.028 -2.201	**0.173** -1.363	0.046 -1.992
urine familiar ♀	5.8 ± 4.8	10.8 ± 6.2	9.8 ± 6.0	10	0.008 -2.666	**0.799** -.255	0.053 -1.938
urine stranger	3.5 ± 2.5	10.9 ± 7.2	8.5 ± 8.7	19	0.000 -3.702	**0.107** -1.610	0.000 -3.562
urine stranger ♂	3.7 ± 3.3	10.0 ± 7.3	6.3 ± 4.2	5	0.043 -2.023	0.042 -2.032	0.043 -2.023
urine stranger ♀	3.4 ± 2.3	11.2 ± 7.4	9.3 ± 9.9	14	0.002 -3.107	**0.433** -.785	0.003 -2.983
control	7.2 ± 4.6	6.0 ± 3.9	6.0 ± 3.5	6	0.249 -1.153	0.753 -0.314	0.463 -0.734

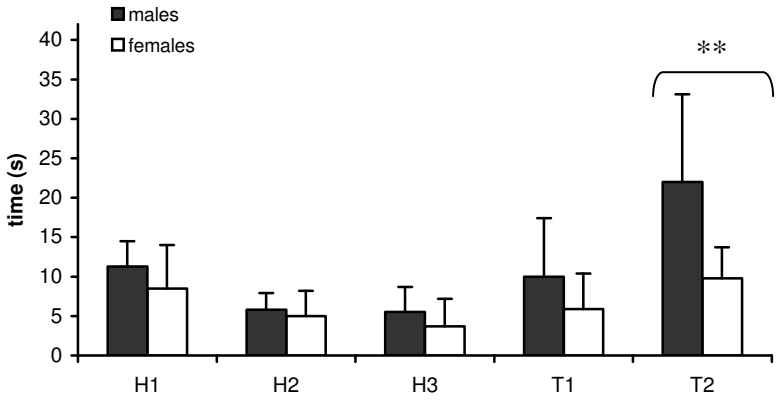


Fig. IV.1.4: Mean (± SD) times that male (grey columns) and female (white columns) subjects spent investigating **ano-genital odours** in the habituation-generalization test (**familiar condition**). The first three columns represent three consecutive habituation trials (H1-3, during which the subject was presented the ano-genital odour of sibling one) followed by two consecutive test-trials. During test one = T1 the odour of a second sibling was presented to the subject; the odour in test two = T2 came from a stranger. Significance level $p < 0.05$, ** = $p < 0.01$, s = seconds.

Tab: IV.1.4: Mann-Whitney *U*-test for differences in the mean time of sniffing at the odour in the test-trial two (T2), demonstrating differences between males and females sniffing at the ano-genital odour and urine odour during the familiar condition respectively; Differences of sniffing times at urine odour between the familiar condition compared to strangers' condition according to the subjects sex (male and female). Significance level $p < 0.05$, s = seconds.

test	$\bar{x} \pm$ SD	n	U	p
ano-genital familiar ♂ and ♀	♂ = 22.0 ± 11.0 s	♂ = 9	12.000	**0.006**
	♀ = 9.7 ± 3.9 s	♀ = 10		
urine familiar ♂ and ♀	♂ = 20.1 ± 18.6 s	♂ = 6	18.000	0.193
	♀ = 9.8 ± 6.0 s	♀ = 10		
urine familiar and stranger ♀	fam. = 13.6 ± 12.8 s	fam. = 16	94.000	0.055
	str. = 8.5 ± 8.7 s	str. = 19		
urine familiar and stranger ♂	fam. = 20.1 ± 18.6 s	fam. = 6	4.000	**0.045**
	str. = 6.3 ± 4.2 s	str. = 10		

Similarly to the familiar condition, all subjects (males and females similarly) in the strangers' condition habituated to the presentation of the ano-genital odour of a stranger in three consecutive trials (Friedman's rank test: $\chi^2 = 24.947$, $p = 0.000$, $df = 2$, $n = 19$).

In contrast to the familiar condition subjects did not increase their investigation when the odour of a second stranger (sibling of the first one) during T1 was presented

(Wilcoxon-rank-test: Z = -1.363, p = 0.173, tab. IV.1.3; fig. IV.1.5, below). They generalized between those two odours.

However, significant increase was found in the sniffing time at the odour from a third stranger, the novel stranger that came from a different family during the second test-trial (Wilcoxon-rank-test: Z = -3.472, p = 0.001; tab. IV.1.3, fig. IV.1.5, below).

No noticeable differences were found between the first odourous contact during the first habituation trial to the sibling one in the familiar condition (4.6 ± 3.4 s) compared to the strangers' condition, when the first stimulus came from the stranger one (5.4 ± 4.5 s, cf., tab. IV.1.3).

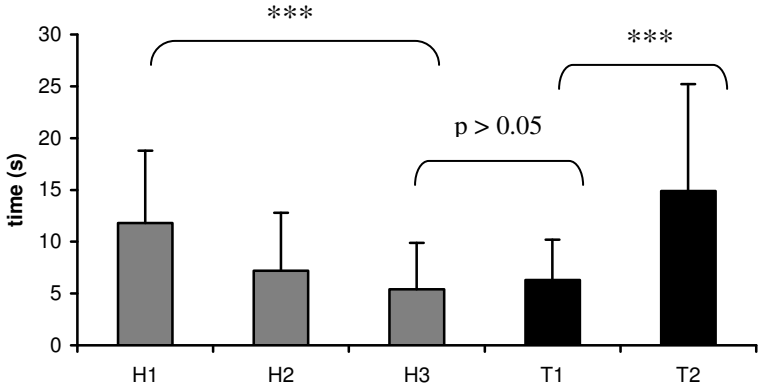


Fig. IV.1.5: Mean (± SD) times that subjects spent investigating **ano-genital odours** in a habituation-generalization test (**strangers' condition**). During the habituation phase (H1-H3), the ano-genital odour of stranger one was presented in three consecutive trials. In test one = T1 the ano-genital odour of the stranger two (a sibling of stranger one) was used. In the following test two = T2, the odour of the novel stranger (not related to stranger one and two) was presented. Significance level $p < 0.05$, *** = $p < 0.001$, s = seconds.

Urine odour

Presenting urine as odour stimulus leads to comparable sniffing times (5.5 ± 3.9 s) during the first contact as presenting of ano-genital odour (4.6 ± 4.3 s); irrespective of familiar or strangers' condition (tab. IV.1.3 above). Thus, no difference between these both odour sources was found.

Habituation occurred in the **familiar condition** throughout consecutive test-trials (Friedman's rank test, χ^2 =21.500, p = 0.000, df = 2, n = 16; tab. IV.1.3).

Mole-rats showed significant increase in the mean time of sniffing during the first test-trial, when they were exposed to the second sibling, compared to the third habituation trial (Wilcoxon-rank-test, p = 0.001, Z = -3.408; tab. IV.1.3, fig. IV.1.6 below).

The time subject spent investigating the odourized plate during the second test-trial (i.e. urine odour of the stranger) was, however, only slightly increased in comparison to the first test-trial, when the odour came from the second sibling, which contrasts the

findings obtained by ano-genital odour testing (Wilcoxon-rank-test, p = 0.605, Z = 0.517; tab. IV.1.3 above, fig. IV.1.6 below).

Females and males differ, which becomes obvious by comparing the first and the second test-trial (presentation of the urine odour of the second sibling and then of the stranger). Males sniffed on average 20.1 ± 18.6 s, whilst females sniffed only 9.8 ± 6.0 s. This result was, however, not significant, which might be caused due to large SD in males (Mann-Whitney U test: p = 0.193, U = 18.0; tab. IV.1.4 above, fig. IV.1.7 below).

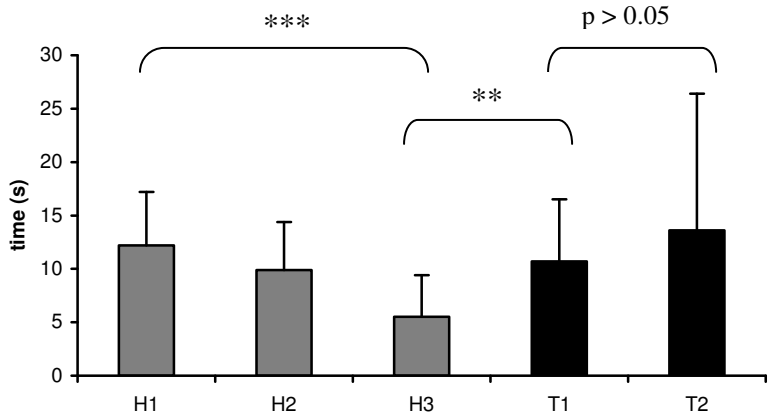


Fig. IV.1.6: Mean (± SD) times that subjects spent investigating urine odours in a habituation-generalization test (**familiar condition**). The first three grey columns represent three consecutive habituation trials (H1-3, during which the subject was exposed to the **urine odour** a sibling) followed by two consecutive test-trials (black columns). During test one = T1 the odour of the second sibling was presented to the subject; the odour in test two = T2 came from a stranger. Significance level $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, s = seconds.

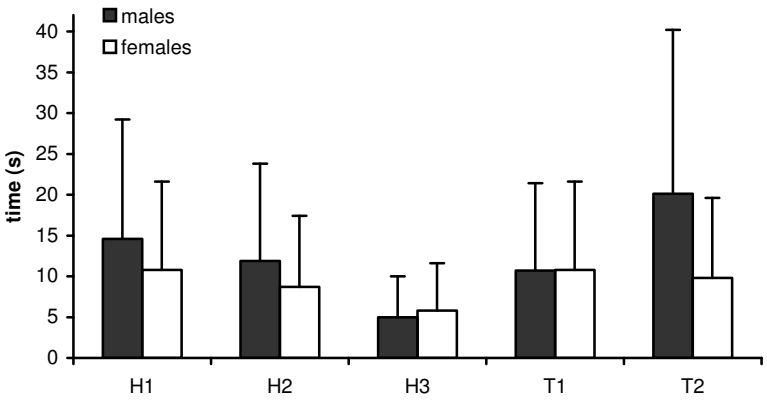


Fig. IV.1.7: Mean (± SD) times that male (grey columns) and female (white columns) subjects spent investigating urine odours in a habituation-generalization test (**familiar condition**). The first three columns represent three consecutive habituation trials (= H1-3, during which the subject was presented the **urine odour** of a sibling) followed by two consecutive test-trials. During test one = T1 the odour of a second sibling was presented to the subject; the odour in test two = T2 came from a stranger. Significance level $p < 0.05$, s = seconds.

The **strangers' condition** delivered some deviations from the results of tests with urine as odour source. Habituation occurred throughout three consecutive trials in males and females likewise (Friedman's rank test: $\chi^2 = 24.827$, p = 0.000, df = 2, n = 19).

In contrast to the strangers' condition with ano-genital odour, mole-rats spent significantly more time investigating the urine odour of the second stranger (sibling of the first one) during test-trial one (Wilcoxon-rank-test, Z = -3.702, p = 0.000), but did not significantly increase their investigation of the odour of the third stranger (the novel stranger, originating from a different family than the first two strangers) during the second test-trial (Wilcoxon-rank-test, Z = -1.610, p = 0.107; fig. IV.1.8 below).

Separating the data clarifies, that in males the expected increase in sniffing time inverted to a significant decrease from 10.0 ± 7.3 s to 6.3 ± 4.2 s, when they were confronted to the odour of the novel stranger (Wilcoxon-rank-test: Z = -2.032, p = 0.042). The decrease was less pronounced in females (Wilcoxon-rank-test: Z = 0.785, p = 0.433; cf., tab. IV.1.3 above).

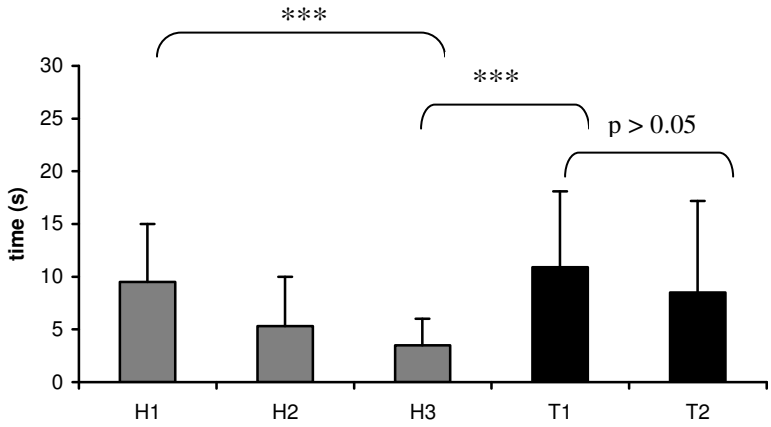


Fig. IV.1.8: Mean (± SD) times that subjects spent investigating **urine odours** in a habituation-generalization test (**strangers' condition**). The first three grey columns represent three habituation trials (H1-3) during which the urine odour of an unknown, unrelated individual ("stranger one") was presented. In test one = T1, the urine odour of the "second stranger" (sibling of the first stranger) was used. In the following test two (T2) we presented the odour of the "novel stranger" (from a second strange family). Significance level p < 0.05, *** = p < 0.001, s = seconds.

Thus, whilst in the familiar condition there was a clear trend of the subjects to increase the investigation time from test-trial one to test-trial two, the strangers' condition delivers reversed data. In this respect, in the strangers' condition *Fukomys* mole-rats decreased their sniffing time when the urine-odour of the "novel" stranger from a second family was presented to them.

However, this trend was not significant, compared to the second test-trial between familiar and strangers' condition (Mann-Whitney *U*-test, p = 0.055, U = 94.000; tab. IV.1.4).

Anyhow, the males showed stronger interest in a stranger compared to females, since they sniffed significantly longer at its odour in the familiar condition than in the strangers' condition (Mann-Whitney *U*-test, p = 0.045, U = 4.000; tab. IV.1.4 above).

IV.1.3.2 Preference tests

Male preference for different types of female odours

The male preference for odours of the queens (compared to the control plate) was always significant in all types of test odours in both categories of tested males (non-reproductive males, nrm and kings, K) which means that the odour of the queen was always preferred. However, none of the tested odours of the nrf was significantly different to the control plate in both categories of tested males (nrm and K).

Non-reproductive male preference for different types of female ano-genital odours

The Kruskal-Wallis ANOVA test is significant (p = 0.000) according mean times non-reproductive males sniffed at the **ano-genital odour** from the queens (Q) in comparison to the non reproductive females (nrf). The subsequent post-hoc test revealed that males of this category sniffed significantly longer at the ano-genital odour of the queen (26.3 ± 16.1 s) compared to the ano-genital odour of nrf (11.8 ± 7.8 s) and also compared to the control plate (7.7 ± 4.2 s; post-hoc: both p = 0.000; tab. IV.1.5, fig. IV.1.9). Thus the queens' odour was clearly preferred.

Tab. IV.1.5: Kruskal-Wallis ANOVA and **post-hoc-test** (Scheffe) of sniffing times between queens (Q) and non-reproductive females (nrf), Q and control (c), nrf and c. Ano = ano-genital odour, u = urine odour, mix = mixed odour of urine and ano-genital odour. Significance level $p < 0.05$, s = seconds.

	odour source	$\bar{x} \pm SD$ [s]			(n) of each male category	p Kruskal-Wallis ANOVA	post-hoc p		
		Q	nrf	c			Q+nrf	Q+c	nrf+c
nrm	ano	26.3 ± 16.1	11.8 ± 7.8	7.7 ± 4.2	20	**0.000**	**0.000**	0.000	0.490
	u	15.1 ± 10.0	11.9 ± 8.4	7.5 ± 4.1	18	**0.045**	0.491	0.022	0.255
	mix	22.1 ± 16.6	12.5 ± 6.9	6.4 ± 3.2	19	**0.000**	**0.026**	0.000	0.212
king	ano	18.4 ± 16.0	8.6 ± 4.2	6.2 ± 3.4	9	0.172	0.130	0.049	0.879
	u	11.6 ± 4.9	7.1 ± 2.9	5.3 ± 4.2	9	**0.012**	0.090	0.013	0.649
	mix	11.2 ± 6.2	9.0 ± 5.5	4.3 ± 2.9	8	**0.049**	0.714	0.045	0.201

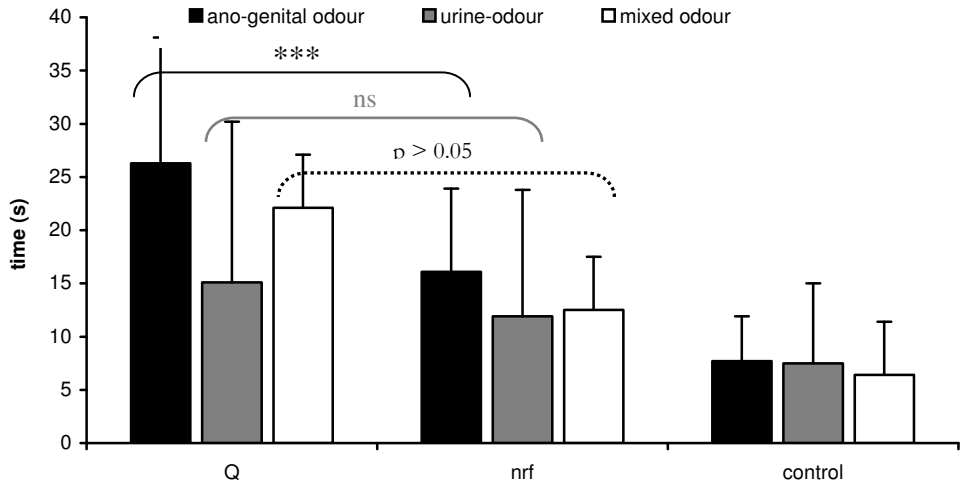


Fig. IV.1.9: Differences between mean times that **non-reproductive males** (nrm) sniffed at three different odour sources (ano-genital odour, urine odour and the mixed odour = mix of both odours) from queen (Q) and non-reproductive females (nrf). Significance level $p < 0.05$, *** $p = < 0.001$, ns = not significant, s = seconds.

Non-reproductive male preference for different types of female urine odours

Likewise, the Kruskal-Wallis ANOVA test is significant ($p = 0.000$) with respect to mean times non-reproductive males sniffed at the **urine** odour from the queens (Q) in comparison to the non-reproductive females (nrf) (Kruskal-Wallis ANOVA: $p = 0.045$; tab. IV.1.5 above), whilst the post-hoc test could not reveal any significant tendency for the preference to the urine odour of the queens (Q) (15.1 ± 10.0 s) over the urine odour

of non-reproductive females (nrf) (11.9 ± 8.4 s, post-hoc test, nrf+Q: p = 0.491; nrf+c: p = 0.255). Nevertheless, there was a clear trend to sniff longer at the urine odour of the queen and thus to prefer their urine odour over that of her daughters (tab. IV.1.5, fig. IV.1.9 above).

Non-reproductive male preference for different types of female mixed odour

Non-reproductive males preferred mixed odour (mixed odour of ano-genital + urine) of the queens (22.1 ± 16.6 s) over those odours from the non-reproductive females (12.5 ± 6.9 s; post-hoc: p = 0.026). The differences were significant (Kruskal-Wallis ANOVA: p = 0.000; tab. IV.1.5, fig. IV.1.9 above).

Kings' preference for different types of female ano-genital odours

Kings did not significantly differentiate between the ano-genital odour of the queens (18.4 ± 16.0 s) and that of non-reproductive females (8.6 ± 4.2 s; Kruskal-Wallis ANOVA: p = 0.172; post-hoc: p = 0.130). Nevertheless, there was a clear tendency to prefer the odour of the queens (tab. IV.1.5 above, fig. IV.1.10 below).

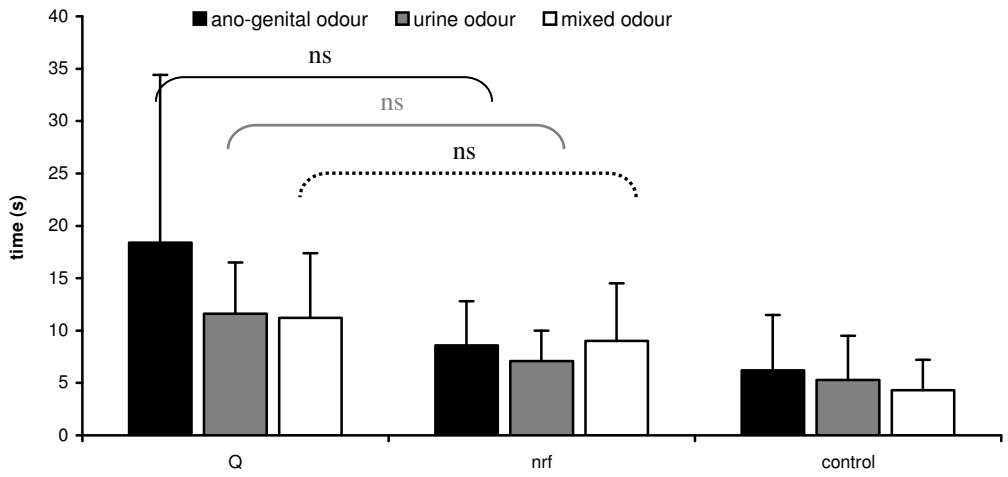


Fig. IV.1.10: Differencees between mean time that **kings** sniff at three different odour sources (ano-genital odour, urine odour and the mixed odour = mix of both odours) from queens and non-reproductive females. Significance level p < 0.05, ns = not significant.

Kings preference for different types of female urine odour

Kings tend to prefer urinary odour of the queens (11.6 ± 4.9 s) over that of non-reproductive females (7.1 ± 2.9 s). This difference was almost significant (Kruskal-Wallis ANOVA: p = 0.012; post-hoc: p = 0.090; tab. IV.1.5 above, fig. IV.1.10).

Kings preference for different types of female mixed odour

Kings tended to prefer the mixed odour of the queens (11.2 ± 6.2 sec) over that of non-reproductive females (9.0 ± 5.5 s), the difference being, however, not significant (Kruskal-Wallis ANOVA: p = 0.049; post-hoc: p = 0.714; tab. IV.1.5 above, fig. IV.1.10).

In sum, the odour of strange queens seem to be more attractive to all categories of males than the odour of their non-reproductive daughters, in all types of odour sources. Ano-genital odour and the mixed odour showed more clear results in non-reproductive males. Urine odour of the queen was almost significantly preferred by kings.

Comparison between three odour sources

None of the three odour sources, ano-genital odour, urine odour or the mixed odour was preferred. In tendency, however, the ano-genital odour seemed to be more interesting for non-reproductive males: they sniffed longer at it (26.3 ± 16.1s) than at the urine odour (15.0 ± 10.0 s; Kruskal-Wallis ANOVA: p = 0.060; post-hoc p = 0.070). The mixed odours got a medium position (22.1 ± 16.6s) which is merely a tendency (tab. IV.1.6, fig. IV.1.11).

This holds also true for kings who tended to sniff longer at the ano-genital odour than at urine odour or the mixed odour, the difference between both latter odour types being negligible.

Comparison between kings and non-reproductive males

Non-reproductive males were in tendency more interested in the (especially ano-genital) odours of strange queens than kings were, even though not at significant level. They sniffed on average 26.3 ± 16.1 s at the queens odour whereas kings sniffed 18.4 ± 16.0 s, (Mann-Whitney p = 0.153; tab. IV.1.7 below).

Tab. IV.1.6: Kruskal-Wallis ANOVA and **post-hoc-test** (Scheffe). Comparison of sniffing times at the **three different odour sources**. Ano = ano-genital odour, u = urine odour, mix = mixed odour of urine and ano-genital odour. Significance level $p < 0.05$, s = seconds.

test-animals	$\bar{x}$ ± SD [s]			n	p Kruscal-Wallis ANOVA	post-hoc p		
	ano	u	ano +u			ano+u	ano+mix	u + mix
nmr	26.3 ± 16.1	15.0 ± 10.0	22.1 ± 16.6	57	0.060	0.070	0.670	0.352
kings	18.4 ± 16.0	11.6 ± 4.9	11.2 ± 6.2	26	0.838	0.411	0.388	0.996

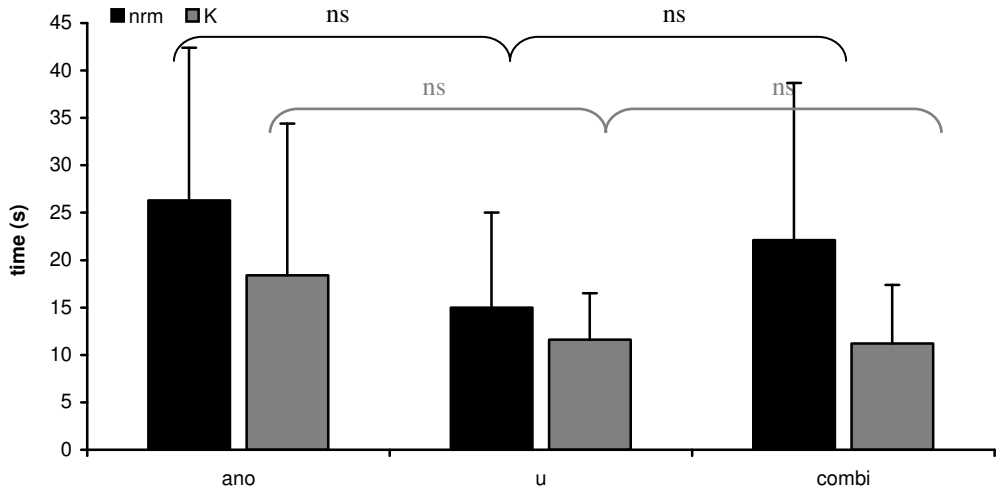


Fig. IV.1.11: Sniffing times at three **different odour sources**. Ano = ano-genital odour, u = urine odour, mix = mixed odour of urine and ano-genital odour. Significance level $p < 0.05$, ns = not significant, s = seconds.

Tab. IV.1.7: Mann-Whitney *U*-test. Comparison in sniffing times at the queen-odours in non-reproductive males (nrm) compared to kings (K). Ano = ano-genital odour, u = urine odour, mix = mixed odour of urine and ano-genital odour. $p > 0.05$ = not significant, s = seconds.

test-animal	n	$\bar{x}$ ± SD [s]			Mann-Whitney p		
		ano	u	ano+u	ano	u	mix
nmr	28	26.3 ± 16.1	15.0 ± 10.0	22.1 ± 16.6	0.153	0.631	0.106
k		18.4 ± 16.0	11.6 ± 4.9	11.2 ± 6.2			

IV.1.4 DISCUSSION

To increase the sample size, findings in the two species and their hybrids were pooled. It should be pointed out, however, that species have never been mixed within one experiment, i.e. subjects were presented exclusively the odours of their own species. Since these sibling species can be distinguished only on the basis of their genetic traits and geographic distribution, but could not be (thus far) distinguished morphologically and behaviourally (Burda 1999), this procedure should be justified.

IV.1.4.1 Habituation-generalization test

Ano-genital odour

Fukomys mole-rats discriminated spontaneously between of own siblings on the basis of ano-genital odours. The pronounced increase in sniffing times during test two in the familiar condition, during which the odour of a stranger from a different family was presented, (fig. IV.1.3) indicates that subjects treated the odours of siblings as similar in comparison to odour of a member of another family, even though they are discriminated. This provides further evidence for odour-genes covariance (i.e. the genetic relationship is reflected by the similarity of odours), and corroborates also previous findings in giant mole-rats (cf., Heth et al. 2002). Strikingly and in contrast to the study of Heth et al. (2002) in giant mole-rats, male small (i.e., Ansell's and Kafue) mole-rats were significantly more interested in the odour of a stranger than females (tab. IV.1.5). This is of particular interest because there are no apparent differences in the social structure and division of labour between giant and small mole-rats. One may speculate that in the studied small mole-rat species, males are more prone to infiltrate strange families and thus males are also more sensitive to recognize and combat the male intruders. Nevertheless, we should keep in mind that also females showed significantly increased interest during the second test-trial when the odour of a strange female was presented.

In the strangers' condition however, subjects did not discriminate between ano-genital odours of strange siblings. This is in consistence with results of previous studies in some other rodent species which were also not able to discriminate odours of unfamiliar siblings, either "forgotten siblings" (after cross-fostering) or unrelated siblings (Todrank et al. 1998 and 1999), or members of another family (Heth et al. 2002), fig. IV.1.5. Such findings were explained as being caused by familiarity (Heth et al. 2002), which enables individuals through previous encounters to associate the odour template with an appropriate individual and match it with the new encountered template. This enables subjects to detect subtle odour differences. If individuals did not have

opportunities to learn the odour template of unfamiliar conspecifics, the odours of those individuals are treated as similar since related individuals have similar qualities of their odours. Subtle differences between them, therefore, cannot be discriminated without previous learning. The strong increase of sniffing during the test-trial two underlines this finding. It demonstrates that *Fukomys* mole-rats are able to differentiate between odours of two unrelated strangers, presumably because their difference is pronounced since they are not related.

In this study it could be demonstrated that *Fukomys* mole-rats are able to discriminate own siblings on the basis of ano-genital odour. They showed the ability to differentiate between their own sibling and a stranger but were not able to distinguish between the odours of two sibling strangers. They "considered" them to be similar, presumably reflecting the odour-genes covariance. This is consistent with the fact that mole-rats were able to differentiate between odours of two unrelated strangers. These findings are in accordance with studies on giant mole-rats (Heth et al. 2002).

Urine odour

Urine odour seems to differ in many ways from the ano-genital odour. After successful habituation mole-rats treated the odour of their own siblings as different from each other (they increased sniffing in course of the third habituation trial to the first test-trial in the familiar condition, fig. IV.1.6). However, the increase of sniffing during the second test-odour (a stranger) was not significant. Urine odours, therefore, seem to provide the basis for differentiating between own siblings, like it was the case with ano-genital odour. On the other hand, urine odour seems not to provide information regarding similarities between siblings. Astonishing and paradox was the finding that mole-rats did not differ between the odour of own sibling and a stranger, whose odour quality should differ intensively (according to odour-genes covariance). Moreover it seems, that much more subtle odour differences could be detected even in the strangers' condition, were the urine odours of two unknown siblings of a different family were compared (fig. IV.1.8). These facts lead me to the conclusion that the hypothesis of odour-genes covariance cannot be applied to urine odour in mole-rats: neither similarity between siblings nor differences between two (unrelated or sibling) strangers seem to be detected on the basis of this source of odour. Alike in the test with ano-genital odour, males tended to be more motivated to sniff during the second test-trial, which nevertheless was not significant and may not have any meaning in this context (tab. IV.1.4).

In the strangers' condition mole-rats treated the odour of two sibling strangers as different to each other (fig. IV.1.8) although their odour quality should be very similar. This contrasts the experiment with ano-genital odour, where subtle differences between

sibling strangers could not be detected. Urine odour, thus, did not allow the subjects to generalize between those odours. Moreover, they could not differentiate between urine odours of two strangers (unrelated with each other). Rather than to increase, they decreased sniffing times during the second test-trial, which was significantly lower in males than in females (tab. IV.1.4). Thus similarities between the odours of sibling strangers could not be detected, whereas their (expectedly very subtle) differences were revealed on the basis of urinary odours. Once again, I conclude that urine odours in *Fukomys* mole-rats do not comply with the hypothesis of odour-genes covariance. Either urine odours of close relatives do not contain information of genetic similarity (rather pronounced individual differences, which seems not quite logical), or since subjects do not have the chance to associate urine odours with its appropriate donor they are not able to prepare an odour template and match it then after. In contrast to ano-genital odour, urine is deposited in the common latrine and may be used to mark objects but does not stick at the individuals' body which makes it impossible to learn a urine based odour template of an individual. The self-reference and phenotype matching process thus are probably not involved in this source of odour. In summary, urine odours seem not to be an appropriate odour source for individual recognition (cf., chapter IV.3.1 and IV.4.1) and may include other information which is important in other social contexts, e.g. biological states like reproductive activity or diet.

Comparing the familiar and strangers' condition, the difference during the second test-trial shows a trend in a higher sniffing times within the familiar condition. In fact, since the purpose of the second test odour is to rule out low motivation levels and to demonstrate similarities between siblings in comparison to a stranger (Heth et al. 2002), it is probable that this result is caused mainly by males through their decreased motivation to sniff at the odour of the third stranger. If urine odour does not deliver enough information about individuals' identity, multiple expositions to urine of different strange same-sex individuals might decrease their interest. Strange same-sex odour may be all the same to the subject. It is irrelevant whether the strange odour comes from a stranger "A" or a stranger "B". Information about the familiarity (known or unknown) and/or relationship (related or not) of the odour donor should be enough to react appropriately. As discussed below, urine may contain different information, e.g. related to sex or the reproductive status of an individual. Unquestionably, judging from demonstrative marking behaviour, urine seems to be of importance for *Fukomys* mole-rats.

IV.1.4.2 Preference test

The results demonstrate first, that in the ano-genital odours of the reproductive females, the queens, differ from those of non-reproductive females, the daughters. Second, queens' odours in tendency are more attractive to strange non-reproductive males as well. Even to males, the control plate were of comparable interest compared to all kind of odours presented from daughters, demonstrating the low impact of daughter odours to the subjects. Although, however, reproductive males (kings) did not show preference for any of the presented odours, it does not necessarily mean that they were not able to detect odour differences between a strange queen and her daughter. Instead, the reason may be found in their low motivation levels since they are already "engaged".

Urine odours from queens seem also to arouse more interest than those of daughters, which thus probably differ in quality between both female categories likewise. Even if the ano-genital odour delivered the clearest results, none of the three odour sources, ano-genital odour, urine odour or the mixed odour was preferred on significance level. In tendency, however, the ano-genital odour seemed to be that one with the strongest behavioural impact on males. It should be noted here, that the sample size of kings was the half compared to those of non-reproductive males, which may have been resulted in a more ambiguous outcome, e.g. not clear statistically significant preference of queens ano-genital odour but at the same time higher mean sniffing times at ano-genital odour compared to urine odours (cf., tab. IV.1.5 and 1.7). In sum, also this male category seems to prefer queens over their daughters and concordantly to be more aroused from ano-genital odours than from urine odour or mixed odour.

Instead to get a supposed amplification effect through mixing both kinds of odours, this source of odour got a medium position which is merely a tendency (tab. IV.1.6, fig. 1.11). The impact of ano-genital odour seems somehow to be diluted by urine, since e.g. non-reproductive males sniffed longer at the ano-genital odour than at the mixed odour of the queens but simultaneously shortest at urine.

General conclusions and arising open questions

The hypothesis was that besides individual identification (as was demonstrated for ano-genital odour by habituation-generalization test) other relevant information is encoded in mole-rats odour of different biological states, e.g. reproductive activity. It is not surprising that it could be shown here by using the preference test, that odours of queens are different from those of non-reproductive females, since their reproductive metabolism must differ. The question is, to which extent this difference is reflected in the odourous phenotype of both female categories and which meaning it may have for male *Fukomys*

mole-rats. Moreover, the differences between queens' and daughters' reproductive mechanisms, e.g. spontaneous-, primed estrus or even suppression of estrus and steroidal levels, should be of high interest. If there are different levels of steroids because of altered reproductive activity, it should not be surprising to find odour compounds, typical for queens or daughters in the ano-genital and/or urinary odour or an odourous "bouquet" of a certain proportion of specialized chemical components, which have the power to arouse males' sexual interest. Stepping forward to those open questions, the next three chapters therefore will deal with steroidal and chemo-analytical studies on urine as a first methodological approach. Moreover, urine is an appropriate source to start with, to compare it with the well studied urine proteins, the MUP (major urinary protein) of the model species, the house mouse. Such proteomic analysis will be outlined in the respective chapter.

IV.2 STEROIDS AND REPRODUCTIVE STATUS

IV.2.1 INTRODUCTION

The eusocial structure in *Fukomys* mole-rats results in reproductive skew (cf., general introduction). The female offspring in *Fukomys* mole-rats are, however, not infertile. In fact, Willingstorfer et al. (1998) found in the ovaries of adult non-breeding female Ansell's mole-rats all stages of follicular development up to tertiary follicles. Many unruptured luteinized follicles, yet missing true of corpora lutea, indicated that ovulation was absent. In actively breeding females, however, true corpora lutea were recorded. Apparently, the Ansell's mole-rat is an induced ovulator (Willingstorfer et al. 1998, Burda 1999) like the Natal mole-rat (*Cryptomys*[6] *natalensis*) and the Highveld mole-rat (*C. pretoriae*) (Malherbe et al. 2004). Anovulation may therefore be a result of a lack of (mechanical) stimulation through copulation rather than consequence of reproductive inhibition (Burda 1999).

Chemosignals influence reproductive behaviour and neuroendocrine function in many rodent species, e.g. in mouse (Vandenbergh 1969, Novotny et al. 1986, Mucignat-Caretta et al. 1995, Wersinger & Rissman 2000, Brennan 2004), prairie vole (Williams et al. 1992, DeVries et al. 1997), pine vole (Solomon et al. 1996, Schwab et al. 2004), and hamster (Reasner & Johnston 1988, Lai et al 1994). The urinary odour of unfamiliar breeding males represents an adequate stimulus to accelerate growth and puberty in juveniles in the house mouse (Mucignat-Caretta et al. 1995). In the previous chapter (and see also Heth et al. 2002 and 2004), it was shown that chemosignals are of high relevance in *Fukomys* mole-rats in different contexts, e.g. in reproduction; *F. anselli* and *F. kafuensis* non-reproductive males discriminate and prefer ano-genital odours of reproductive females (queens) to those of non-reproductive females. Moreover, *F. anselli* males prefer ano-genital odours of unrelated females to those of their forgotten sisters and also prefer to mate with unrelated females (Heth et al. 2004).

In order to find out the explanation for the ability of males to recognize queens by urine and especially by ano-genital odour, the basal steroid concentrations (normalized urinary estradiol- and progesterone concentration (mg/Cr)) between three "categories" of females (reproductive queens, rQ; non-reproductive queens, nrQ and non-reproductive females, nrf; cf., chapter IV.1) were compared, before behavioural experiments started. This chapter should contribute moreover to a basic understanding of both, the priming capacity of olfactory stimuli and reproductive activity upon cycling reproductive steroids

[6] Further abbreviated as *C.*

(urinary estradiol- and progesterone concentrations) to monitor indicators of ovulation in sexually inexperienced females. In parallel, vaginal cytology (further termed as colpocytology) was performed in order to find signs of ovulation, with and without olfactory or sexual stimulation. A further goal was, to find an applicable method (on the basis of basal urinary steroid concentrations) to distinguish reproductive queens from non-reproductive females (workers) in order to attribute wild caught animals to their correct family status, since *Fukomys* queens do not morphologically differ from their daughters, contrary to e.g. naked mole rats, *Heterocephalus glaber* (cf., Henry et al. 2007).

A test-procedure consisting of three phases was designed to address the role of olfactory stimuli and of reproductive activity: I. first phase lasting 39 days without manipulation (control), II. second phase of five days with olfactory stimulation of potential reproductive partners, III. third phase lasting five days, during which respective partners were put together and were mated, while steroids and cytological parameters were monitored. Males in which the urinary testosterone concentrations (mg/Cr) were tested over a shorter period of time during all three phases (each lasting five days) were of minor interest. Nevertheless, the impact of olfactory and reproductive activity on urinary testosterone, a cycling reproductive steroid of males, was recorded as well. The mating behaviour was registered qualitatively to elucidate the releasing role of chemical (olfactory) senses in reproductive contexts.

Hormonal regulation of breeding has been studied in South African mole-rats (*Fukomys damarensis* and several *Cryptomys* species) and in naked mole-rats, *Heterocephalus glaber* (reviewed in Bennett et al. 2007), but comparative data on other species of African mole-rats are absent. Thus far, neither hormonal assays nor vaginal cytology, as a marker for ovulation, have been reported for other *Fukomys* mole-rat species but *F. damarensis* (hormonal studies by Snyman et al. 2006). Here, hormones regulating the ovarian cycle were studied in mole-rat species, which have been thus far neglected from the endocrinological point of view – in order to get further insight into relationship between hormonal regulation and olfactory chemical communication.

IV.2.2 MATERIAL AND METHODS

The first part of this study (first test phase) was conducted over a period of 39 days (19/5/2005 to 26/6/2005) so that its duration would encompass at least a part of the estrus cycle, the length of which was estimated as approximately one to one and half month in some bathyergids species (Faulkes et al. 1990, Snyman et al. 2006).

IV.2.2.1 Animals

Three "categories" of females of two sibling species of *Fukomys* mole-rats (*F. anselli and F. kafuensis*) and their hybrids (family Bathyergidae) were studied: (I) reproducing, sexually active queens (rQ), pregnant and/or lactating; (II) non-reproducing queens (nrQ), i.e. dominant, sexually experienced females with a history of breeding which, however, at time of testing, did not show breeding activity like copulating, pregnancy and/or lactation. They had no contact to males at time of the study but they had been successfully reproducing (rQ) before; (III) non-reproductive, and sexually inexperienced female "workers" (nrf). Eleven females (5 rQ, 2 nrQ and 4 nrf) were involved in the control test (first test phase with steroid measurement and colpocytological investigations without experimental behavioural manipulation, described in more detail below). Six of these females (1rQ, 2nrQ and 3 nrf) were involved also in the following two experimental test phases (olfactory stimulation and mating). The reproductively active females were involved only in the control test phase because they already had been active and succesful queens (except for female rQ5). Female rQ5 was wild-caught and its last successful reproduction was one year before the study started, although it copulated regularly with one male (and therefore labelled as rQ). Still she did not monopolize the male (probable king), since another female in the group was mating with it as well. Non-reproductively active females (nrQ and nrf) were involved in all the three test phases, except for female nrf1, because of animal husbandry reasons. The animals of known age were three to fifteen years old (tab. IV.2.1a). All animals were kept under identical conditions (cf., chapter IV.1).

Among the examined males, there were five non-reproducing males (nrm) and one non-reproducing king (nrK1), which copulated regularly with the female and rQ5 (cf., above). The king nrK1 had already a history of breeding. He lost his queen six months ago and still lived reproductively inactive in his family. Since four of the non-reproducing males were caught in the wild, their previous breeding history was not certain, but within their actual families they seemed to be sexually quiescent. One of the non-reproducing males was born and raised in captivity (cf., tab. IV.2.1b).

Tab. IV.2.1a: Characteristics of females involved in steroid analysis and colpocytology. Participation in experimental days during all test phases is given in the fourth column. ***** = during experimental phases, Δ = before the first day of experiment.

number	female	birth/captured	partici-pation (days)	perforated vagina	pregnany *	conception *	lactation *
1	rQ1	wild captured, december 1999	1-39	+	since day 14	day 7	+
2	rQ2	wild captured, december 1999	1-39	+	-	-	+
3	rQ3	born on 24/6/1999	1-39	+	since day 38	-	+
4	rQ4	born on 22/9/1995	1-39	+	since five weeks Δ	-	+
5	rQ5	wild captured, july 2002	1-51	+	-	-	-
6	nrQ1	born on 12/11/1990	1-51	+	-	-	-
7	nrQ2	born in summer 1994	1-32	+	-	-	-
8	nrf1	born on 13/11/2000	1-39	+	-	-	-
9	nrf2	wild captured, july 2002	1-51	+	-	-	-
10	nrf3	born on 9/7/2000, daughter from nrQ1	35-51	-	-	-	-
11	nrf4	wild captured, may 2004	1-32	+	-	-	-

Tab. IV.2.1b: Characteristics of **males** involved in steroid analysis during experimental phase 1-3. Participation in experimental days during all test phases is given in the last column.

number	male	birth/captured	participation(days)
1	nrK1	wild captured, march 2003	35-51
2	nrm1	born on 13/11/2000	36-50
3	nrm2	wild captured, july 2002	35-51
4	nrm3	wild captured, july 2002	38-51
5	nrm4	wild captured, july 2002	35-50
6	nrm5	wild captured, may 2004	35-48

IV.2.2.2 Colpocytology and urine collection

Except for one non-reproductive female (nrf3), all females were subjected to the daily process of vaginal smear at early morning. The procedure was as follows: To avoid contamination, disposable gloves were worn. The animal was picked up gently in that it was held with two fingers on its backside with the head upside down. The cotton bud (prepared of a tooth pick, and soaked with 0.3% NaCl solution) was carefully inserted into the vagina, thoroughly rotated and smeared onto a microscope slide which was then air-dried for at least three min, fixed afterwards for 5 min in methanol, again air-dried and stained for 20 min in a Giemsa-solution (ten drops Giemsa in 10 ml ddH_2O). Each slide was examined at 25 to 100fold magnification by AnalySIS-Systems (AnalySIS pro FIVE, Soft Imaging Systems GmbH, Farb-TV-Kamera Kappa CF15MC und Olympus-microscope VANOX AHBT3). The smear could not be spread evenly on the slide and thus counting different cell types in grids on the slide was not possible. Therefore, a semi-quantitative method for assessment of different cell types and their ratios was established. The amount of cells was classified as follows: stage 1 = solitary/insular, stage 2 = sporadic, stage 3 = few, stage 4 = many, stage 5 = lots of, stage 6 = plentiful cells. Together with inter-stages I got a twelve-stage system ranging from 1 over 1.5 to 6.0 (cf., appendix fig. VII.B1, VII.B3). Since nucleated cells are the most frequent in the smear in mice at proestrus and develop into cornified cells, the aim was to find periodical changes between these two cell types. Estradiol and progesterone influence the cell layers of uterus and the vagina. Therefore, a further aim was to look for correlation firstly, between the estradiol concentration and the amount of nucleated cells and secondly, between the progesterone concentration and the amount of nucleated cells. Cornified cells were found not to be a reliable indicator. Parallel to colpocytology, urine was collected almost daily (see chapter IV.1.2), preferably to measure steroids regularly every second day (which sometimes, however, was not possible). Therefore, it should be noted that steroids could be measured only irregularly, thus possibly masking stronger correlation between those two cell types and estradiol or progesterone concentration. Collected urine was frozen and kept under –20° C until analysis.

IV.2.2.3 Steroids and creatinine assessment

In females, the concentration of estradiol, progesterone and luteinizing hormone (LH) in urine was analysed.

Urine of males was analysed for testosterone concentration. To take different urine concentrations into account, the total protein concentration of each sample had to be corrected for the creatinine concentration (Snyman et al. 2006). Creatinine is a breakdown

product of tissue proteins, usually formed in muscles (Schmidt-Nielson 1997) and excreted at a constant rate. Creatinine (Cr) concentrations were measured on the Bayer ADVIA1650 system (Siemens Medical Solutions Diagnostics, Fernwald, Germany) according to the Jaffé method (reagents supplied by the manufacturer). At least 100 µl of urine were necessary for analysis. Steroids were determined on the Bayer Centaur system (Siemens Medical Solutions Diagnostics, Fernwald, Germany) using immunoassay. Between 500 and 700 µl of urine were required for the analysis. If available, urine of every second day was analysed.

Urinary estradiol, progesterone and LH samples were calibrated to the Bayer Centaur system, since the automatic assays (see above) are optimized to human plasma samples. From each steroid four dilutions (1:5, 1:10, 1:20, 1:50) were measured three times and tested by a regression curve ($R^2 > 0.98$). Distilled water was used for the dilution. For estradiol, the dilution of 1:50 was below the detection limit. Testosterone and creatinine were measured at the same time for a MSc. thesis of Boris Kramer, in which differences in hormones between reproductive and non-reproductive animals were investigated. Different parameters, i.e. influence of storage temperature (–20° C and –80° C), storage time and variation coefficient, were investigated to check this assay (Kramer 2006).

IV.2.2.4 Test-procedure

During the second phase (day 40-45) of the experiment, non-reproductively active partners of opposite sex (six males and females; cf., tab. IV.2.2) were exposed (for six hours each day) to the full olfactory "bouquet", composed of odours of their respective partners including, body secretions, urine and faeces odour in order to reach the highest possible reaction. Before preparing the test-cage with new odour donor, it was consecutively cleaned: first with water, followed by 3 % acetic acid, again with water and then dried with tissue paper. Every evening this process was repeated and each odour donor got the same cleaned and newly prepared cage for the next night. To create an odourous mixture, the respective sex partners were put into a clean glass terrarium with 300 g horticultural peat, two pieces of tissue paper and one carrot for 12 hours overnight in the course of six subsequent nights. Every early morning, the inhabitant of cages was exchanged, i.e. females were put into the cage of a male odour donor and vice versa for six hours (fig. IV.2.1). Thus the subjects were exposed to the odours left from the former inhabitant of that particular cage. After six hours, urine and vaginal smear were collected (see above) and the subjects were removed to their respective family groups for next six hours in order to keep family contact. Before the next night, test-subjects were put into a freshly prepared cage (see above). The glass terrarium of exchange partners were of the same size.

The day following the last olfactory stimulation, the third test phase started. The respective odour partners (tab. IV.2.2) were put together into one cage and the mating behaviour and frequency of copulation within the first half hour was recorded. The first vaginal smear was collected 0.5 h, 4 h, and 10 h after successful mating. Thenafter, vaginal smear was collected twice a day, every twelve hours for a period of five days. Urine was collected from each subject after the mating procedure and then collected daily for five subsequent days.

Ethical note

All efforts were taken to minimize stress of the animals (see chapter IV.1). The animals were tame, and were stressed negligibly by handling for vaginal smear, but obviously not stressed at all by urine sampling.

Fig. IV.2.1: Example of respective mating partners, demonstrating the **experimental setup** during the olfactory stimulation by the former inhabitant in the morning. Female rQ5 and its partner nrm2 are shown.

Tab. IV.2.2.: Characteristics of **(mating) partners** in **test phase 3**. Females are categorized as nrf = non-reproductive females, rQ = reproductive queens and nrQ = non-reproductive queens. Males are categorized as nrm = non-reproductive males and nrK = non-reproductive kings. + = positive identification of mating success or conception, - = negative identification of mating success or conception.

mating partners				
number	**female**	**male**	**mating success**	**conception**
1	rQ5	nrm2	+ , spermatocytes	+
2	nrQ1	nrK1	+ , spermatocytes	+
3	nrQ2	nrm1	+ , no spermatocytes	-
4	nrf2	nrm4	+ , no spermatocytes	-
5	nrf3	nrm5	- , ♂aggression and separation, new partner, nrm6 +, spermatocytes	+
6	nrf4	nrm3	- , no aggression	-

IV.2.2.5 Statistical analysis

Non-parametric statistical tests were used, since data did not fit normal distribution (Kolmogorov-Smirnov-test) and variances were not homogeneous (Levene-test). To test for an increase of urine steroid concentrations in the course of the test phases one to three, I used the Friedman's rank-test. Significant differences of pair wise data were tested by the Wilcoxon-rank-test for paired samples (e.g. mean concentration of urinary estradiol (nmol/mg Cr) in test-period 1 vs. 2, in test-period 2 vs. 3 and in test-period 1 vs. 3. The similar procedure was applied for analyses of urinary progesterone and testosterone). To compare mean steroid concentrations between different categories of subjects (e.g. urinary estradiol concentration of sexually active rQs vs. sexually inactive nrQs and nrfs) during the control phase (phase one), Mann-Whitney *U*-test for independent samples was used. The similar procedure was applied for assessment of urinary progesterone.

The significance level was set to $p < 0.05$. Only outliers from the mean were excluded to produce comparable data. There were partly large differences in the numbers of samples (n) between respective test phases, due to their different length (first test phase = 39 days, second test-phase two = 5 days). All statistical analyses were performed with SPSS (version 11). Since the classification of vaginal discharge was semi-quantitative, statistical analysis was not performed.

IV.2.3 RESULTS

IV.3.1.3 Colpocytology

Eleven females were monitored with respect to colpocytology and steroids in course of olfactory stimulation and subsequent mating in order to find cytological indication of estrus (cf., tab. IV.2.3). The females rQ5, nrQ1, nrQ2, nrf2, and nrf4 were involved in all three test-procedures.

In all the females (but nrf 3) the vagina was perforated and it was possible to obtain vaginal smears and to study vaginal cytology (cf., tab. IV.2.1a). In all the examined females, leucocytes were found, occasionally in high numbers. Differentiation between types of nucleated cells was difficult because parabasal- and intermedial cells were quite similar, and superficial cells were rare. Therefore, this analysis differentiates exclusively between cornified cells, nucleated cells in general, and granulocytes. Granulocytes were always seen in high numbers (stages 4 to 6).

Correlation between different cell types

Within the course of 39 days of the examination period, the numbers of nucleated and cornified cells varied largely in the rQs. Both cell types showed a mutually dependent pattern of changes, i.e. decrease in numbers of nucleated cells leads to increase in numbers of cornified cells and vice versa (tab. IV.2.3, fig. IV.2.2). Among nrfs, however, this pattern was not present in two females, and only partly recognizable in further two individuals (tab. IV.2.3).

No correlation between numbers of nucleated cells and cornified cells was detected in nrQs (tab. IV.2.3). Breeding queens (rQs) exhibited slight periodicity (mean period 5.2 days) in peaks of nucleated cells. Non-breeding females (both nrQs and nrfs, except for nrf2) did not show any periodicity in this respect.

Correlation between colpocytology and steroids

Urinary estradiol concentration had, if any, only slight influence on the cytological picture of nucleated cells or cornified cells in rQs.

An increase in estradiol concentration coincided occasionally with more frequent occurrence of nucleated cells (cf., tab. IV.2.3). This correlation was not found in nrQ1 and nrfs (thus, e.g. nrf2 had frequently estradiol concentrations below the detection limit, yet of the numbers of nucleated cells did not decrease, fig. IV.2.3).

Thus, within the **first phase** of the experiment, before experimental manipulation of females, no sign of spontaneous ovulation based on colpocytology or urinary estradiol could be detected.

The non-reproductive queen nrQ2, however, showed correlation between steroids and cytology, since during the second test-phase, in the suspected proestrus, through olfactory stimulation a pre-stage of estradiol peak occurred, the number of granulocytes decreased, the number of nucleated cells increased, while the numbers of cornified cells remained still at the medium level. During the suspected induced estrus in the mating session in the third test-phase, indicated by a marked estradiol peak (day 43), the number of granulocytes and nucleated cells decreased, yet fluctuated, and the numbers of cornified cells increased up to stage 5. This cytological picture resembles in its tendency roughly a picture of estrus in the laboratory mouse, exclusively dominated by cornified cells, without leucocytes or other cell types. None of the other tested females, however, showed any resemblance to that characteristic cytological picture of proestrus or estrus. Specifically, as already mentioned above, a slide without granulocytes or a slide exclusively with cornified cells was never obtained. Moreover, there were no signs of correlation between nucleated cells and cornified cells to estradiol (except in three rQ) and progesterone, respectively (cf., tab. IV.2.3, fig. IV.2.2, IV.2.3, and appendix fig. VII.B4-7).

During the **second** and the **third phases** of the experiment, no noticeable estrus like changes could be recorded in all categories of females. Nucleated cells were always visible in nrf 2 (cf., fig. IV.2.3). Even successful matings of females rQ5 and nrQ2 did not reflect estrus on level of colpocytology on day 49 and 50, when the estradiol reached its highest peak (fig. IV.2.4; appendix fig. VII.B8)

Altogether, there was no correlation between induced estrus and olfactory stimulus and/or the mating session afterwards. Cytological estrus changes were not detected.

Solely three of the reproductively active queens (cf., tab. IV.2.3) showed a weak periodicity between nucleated cells and cornified cells as well as a weak correlation between high estradiol level and high numbers of nucleated cells and were thus different from reproductively inactive females (nrf and nrQ).

Tab. IV.2.3: Correlation (not statistical) between two cell types (**nucleated cells and cornified cells**, between nucleated cells and **urinary estradiol concentration,** and nucleated cells and **urinary progesterone concentration**). + = correlation, ++ = high correlation, - = no correlation.

female category	nucleated cells and cornified cells		nucleated cells and estradiol		nucleated cells and progesterone	
rQ1	+	+, weak	+/-	+	-	-
rQ2		+		+		-
rQ3		++		-		-
rQ4		+		+		+
rQ5		+, moderate		-		+, moderate
nrQ1	-	-	-	+	-	-
nrQ2		-		-		-
nrf1	-	+, moderate	-	-	-	-
nrf2		-		-		-
nrf4		+		-		+

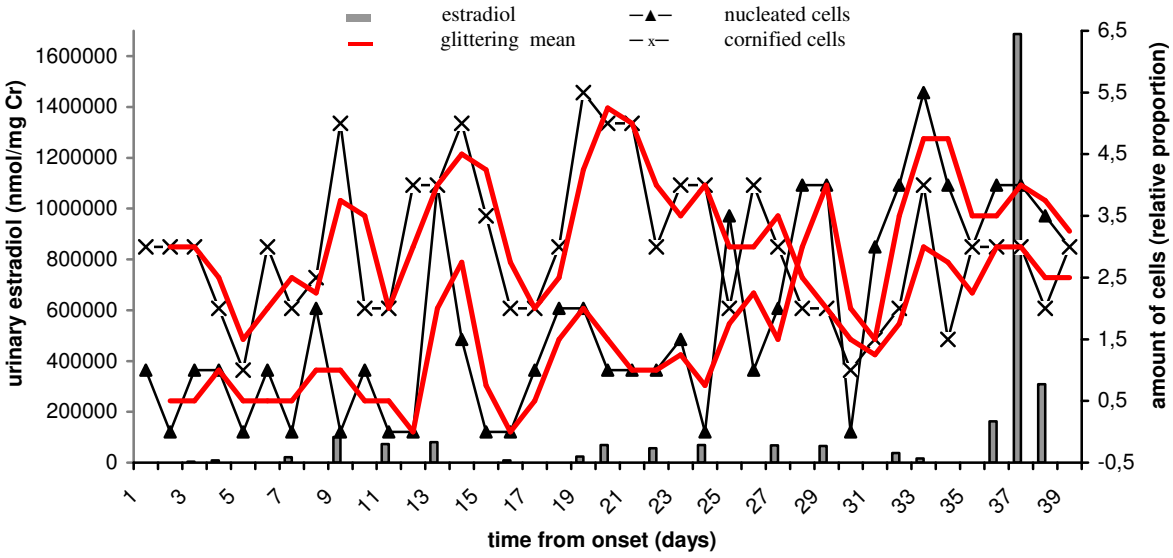


Fig. VI.2.2: Pattern of two cell types (nucleated cells and cornified cells) of vaginal smear in relation to **urinary estradiol concentrations** (nmol/mg Cr) in female **rQ3** during the first test phase. Grey bars are levels of estradiol. Lines with a cross show the amount of cornified cells and lines with triangles show the amount of nucleated cells. The two red lines are glitting means of both cell types.

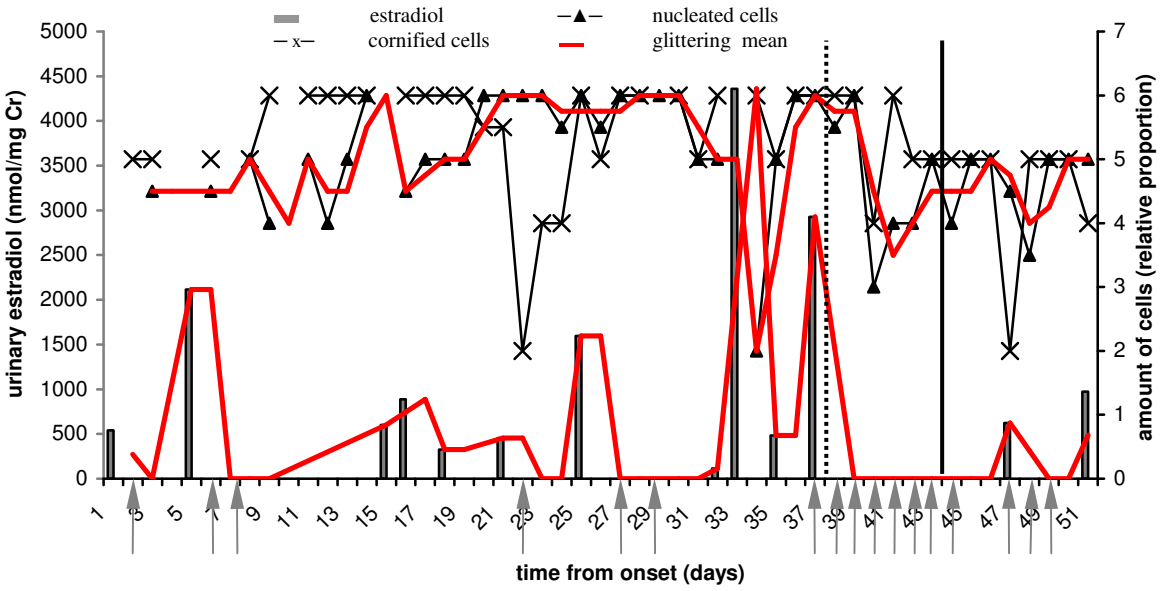


Fig. IV.2.3: Pattern of two cell types (nucleated cells and cornified cells) of vaginal smear in relation to **urinary estradiol concentrations** (nmol/mg Cr) in female **nrf2** during all test phases. Grey bars are levels of estradiol. Lines with a cross show the amount of cornified cells and lines with triangles are the amount of nucleated cells. The two red lines show glitting means of both cell types. Grey arrows indicate estradiol concentration below detection limit, which was 5 nmol/l. ..... Start of the second test phase (olfactory stimulation), ——— start of the third test phase (mating).

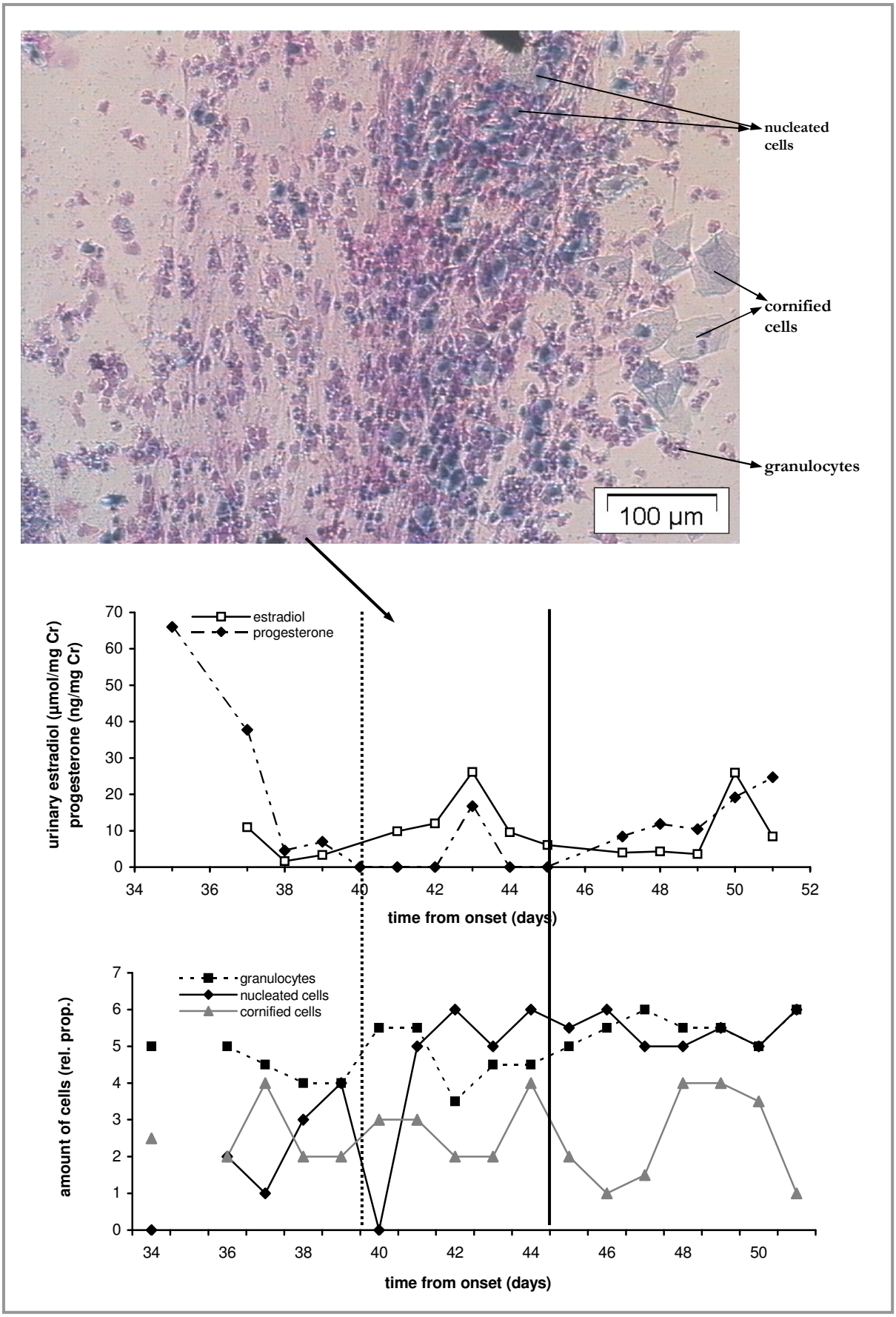


Fig. IV.2.4: Urinary estradiol and **progesterone** concentrations of **nrQ2** in relation to **nucleated cells**, **cornified cells** and **granulocytes**. Test phase one without manipulation until day 39; ····· start of the second test phase (olfactory stimulation), —— start of the third test phase (mating). Above slide shows **vaginal cell populations** of day 41.

IV.2.3.2 Steroids and creatinine assessment

The inter- and intra-assay variations of progesterone and estradiol concentrations were found to be within acceptable limits allowing comparison between urine concentrations of different individuals (cf., appendix fig. VII.B9 and VII.10). From estradiol however, the detection limit was 5 nmol/l. Moreover, low testing ranges (in the range of about 40 nmol/l) of estradiol led to error-proness. Since the regression curve of LH (luteinizing hormone) was not reliable, this steroid was eliminated from further interpretation. The results of creatinine and testosterone concentrations were in acceptable limits according to variation coefficient (creatinine = 11.5 ± 13.2 %, testosterone = 13.3 ± 14.6 %) (cf., Kramer 2006). Lower testing ranges of testosterone (0.35-20 nmol/l) were, however, error-prone. The storage temperature did not influence the testosterone concentration, whereas the storage time was positively correlated with an increasing testosterone concentration. Altogether the testosterone and creatinine values were found to be within acceptable limits (Kramer 2006).

IV.2.3.2.1 Females

Mean steroid concentrations in the first test phase

Mean urinary estradiol concentrations were significantly higher in sexually active females (228,059 nmol/mg Cr) than in sexually inactive females (4,536 nmol/mg Cr; Mann-Whitney *U*-test, sexually active n = 88, sexually inactive n = 41, $p < 0.001$) (fig. IV.2.5, appendix tab. VII.B1).

The mean urinary progesterone concentrations were significantly higher in sexually active females (all rQs = 35.1 ng/mg Cr) than in sexually inactive females (all nrQs and nrfs = 12.9 ng/mg Cr; Mann-Whitney *U*-test, sexually active n = 96, sexually inactive n = 74, $p = 0.001$ (cf., fig. IV.2.5).

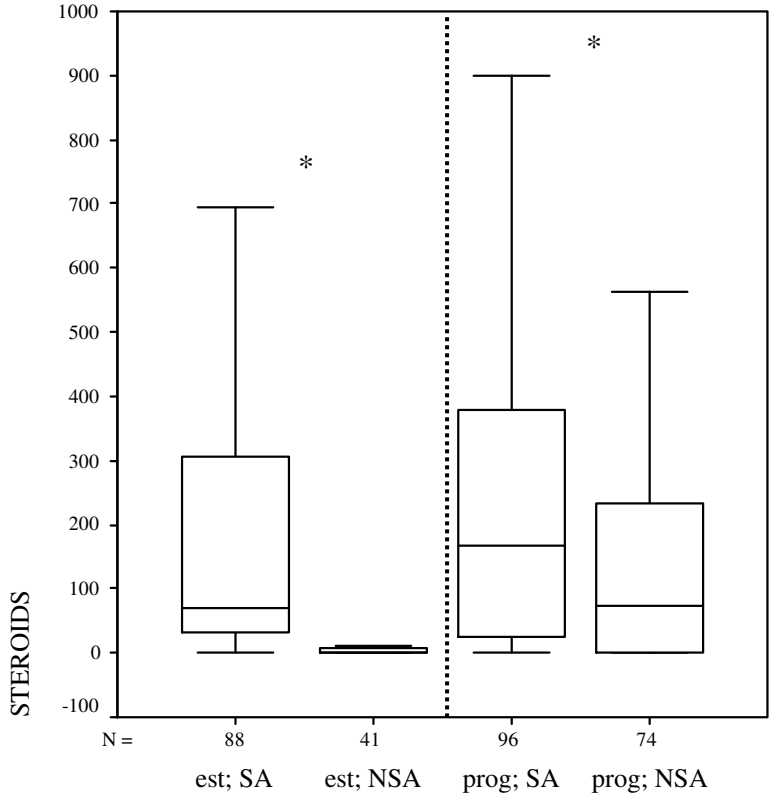


Fig. IV.2.5: Boxplot of **Mann-Whitney *U*-test** for independent samples: **Mean urinary steroid concentrations** (estradiol (nmol/mg Cr) and progesterone (ng/mg Cr*10)) between females of different reproductive activity. SA = sexual active, NSA = not sexual active, est = estradiol, prog = progesterone. Significance level $p < 0.05$, * = $p < 0.001$. N = number of analysed urine samples.

Steroid concentrations in the test phase 1-3

Successful mating resulting in pregnancy and conception was recorded in the females nrQ1 and rQ5. Female nrf3 was attacked by the male in the first mating session but was successfully mated afterwards with a different partner (tab. IV.2.2).

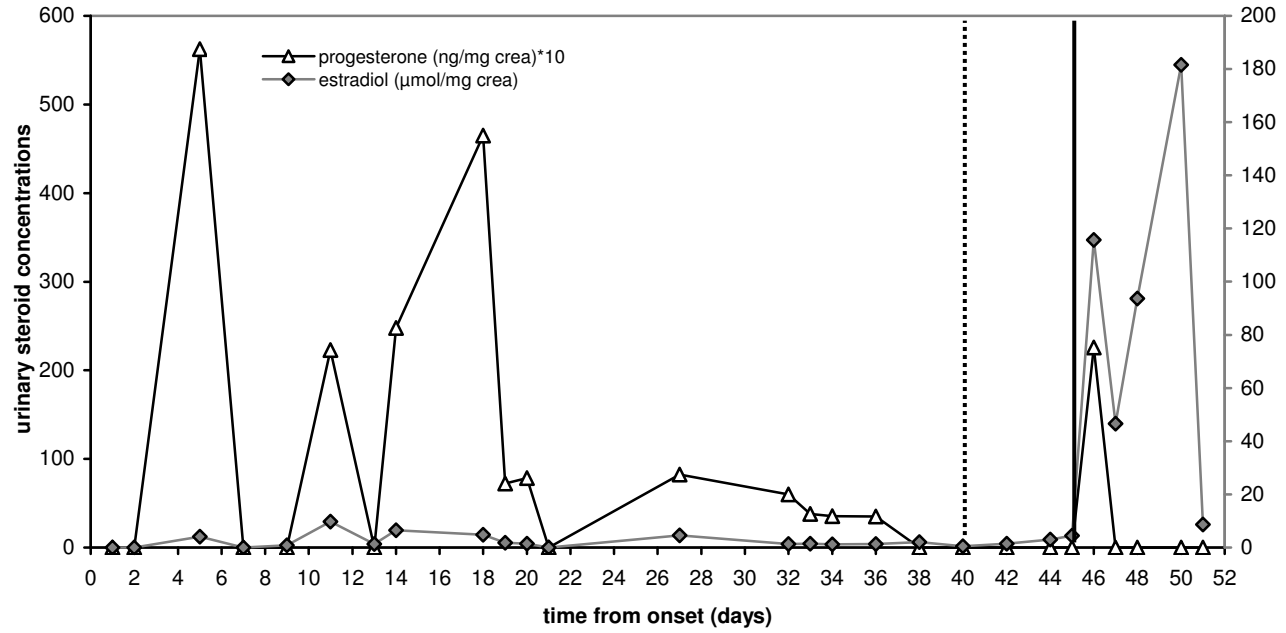
Female **nrQ1** and its male partner nrK1 copulated 51 times within the first half an hour. This pair showed the whole range of mating behaviour (see below). Sperms were found in the first vaginal smear after the first half an hour in huge amount (stage 6, analogous to cell stages in colpocytology). Mating led to pregnancy and conception to two pups 101 days later. The mean estradiol concentration of this female increased after mating significantly ($p = 0.043$, Wilcoxon-rank-test), 37-fold, compared to the control, from 2,355 ± 2,623 to 89,233 ± 66,255 nmol/mg Cr (control phase n = 18, third phase n = 5) (fig. IV.2.6a; cf., appendix tab. VII.B3). Thus, there is a strong correlation for an increase of estradiol during the mating session. Olfactory stimulation (the second test phase) did not affect the mean estradiol concentrations compared to control (Wilcoxon-rank-test: n = 4, $p = 0.068$, $\bar{x} \pm$ SD first phase = 2,355 ± 2,623 nmol/mg Cr,

$\bar{x} \pm$ SD second phase = 2,329 ± 1,754 nmol/mg Cr) (fig. IV.2.6a; cf., appendix tab. VII.B3). The mean progesterone concentration was not influenced by test olfactory stimulation or by mating (Friedman's rank test: n = 4, p = 0.174) (fig. IV.2.6a; cf., appendix tab. VII.B2).

Female **rQ5** showed significant increase (p = 0.043, Wilcoxon-rank-test) in estradiol concentrations following mating, with concentrations rising from 28,407 ± 22,834 to 262,059 ± 93,539 nmol/mg Cr (first phase n = 20, third phase n = 4) (fig. IV.2.7b; cf., appendix tab. VII.B3). The olfactory stimulation of female rQ5 tendentially influenced the estradiol concentration (Friedman's rank test: n = 4, p = 0.05) (tab. IV.2.7b; cf., appendix tab. VII.B2). Although the progesterone increase was not significant (Friedman's rank test: n = 4, p = 0.779) from second test phase to the third test phase there was a tendency towards progesterone concentration increase with a maximum peak on day 48 (Wilcoxon-test: first phase n = 20, second phase n = 4, p = 0.061, $\bar{x} \pm$ SD first phase = 24.3 ± 24.4 ng/mg Cr, $\bar{x} \pm$ SD second phase = 38.9 ± 17.9 ng/mg Cr) (cf., fig. IV.2.6b; appendix tab. VII.B2).

Female **nrf 3** mated successfully with its second partner, yet the concentrations of steroids did not change significantly. Estradiol, on the other hand, increased tendentially from 44 ± 98 ng/mg Cr to 219 ± 536 ng/mg Cr during the olfactory stimulation (Wilcoxon-rank-test: p = 0.661 first phase n = 5, second phase n = 6) (cf., fig. IV.2.8; appendix tab. VII.B3). However, the steroids were measured only for a short period, namely for the last five days of the first test phase.

Anyhow, sexually experienced females, **rQ5** and **nrQ1**, were apparently primed through mating much faster than nrf3 (fig. IV.2.6a,b and IV. 2.7a,b).



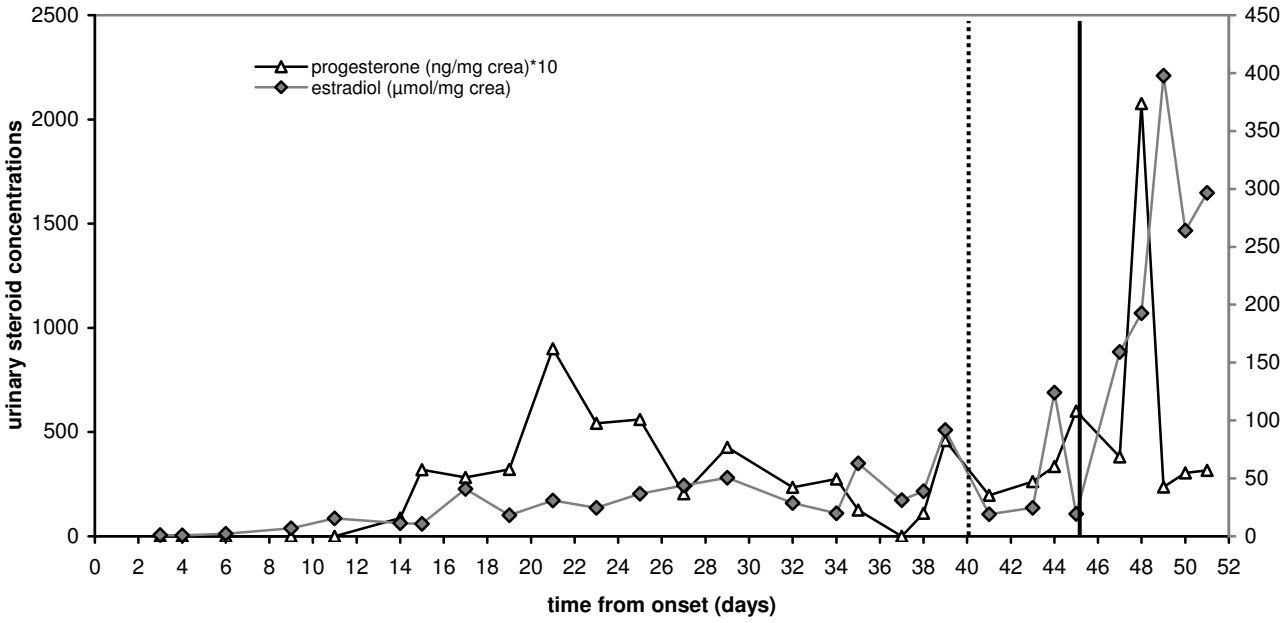


Fig. IV.2.6a,b: Increase of urinary **progesterone** (left y-axis) and **estradiol** (right y-axis) concentrations of females measured during consecutive test-procedures. ····· Start of the second test phase,—— start of the second third phase. Above female **rQ5** and below female **nrQ1**.

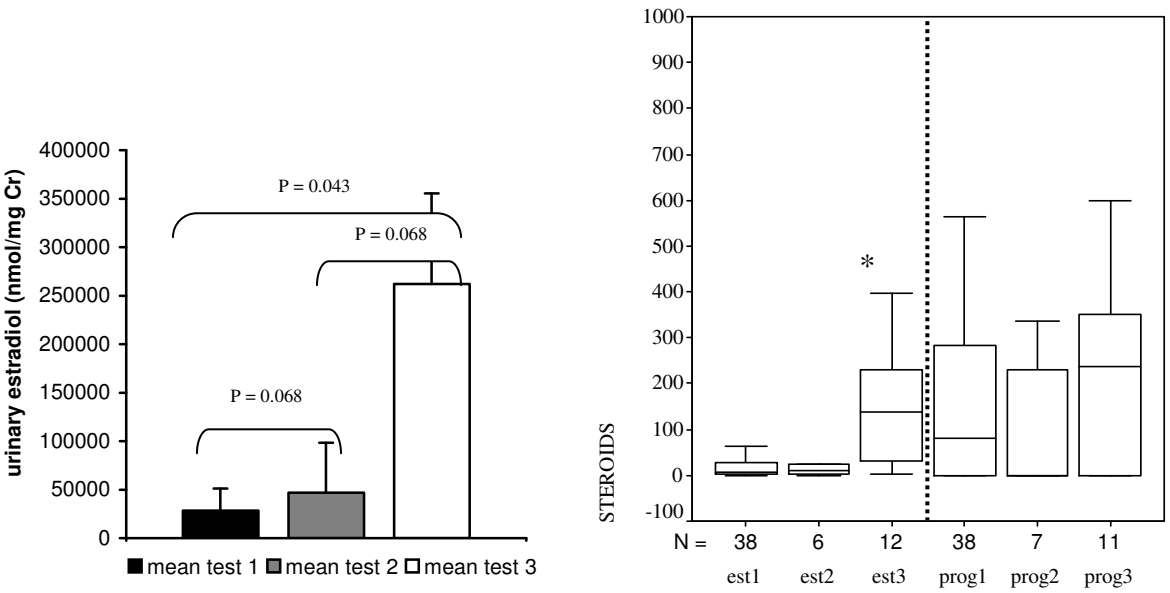


Fig. IV.2.7a,b: Mean concentrations (left) of urinary **estradiol** in **rQ5** measured during consecutive test-procedures (Wilcoxon-rank-test; Significance level $p < 0.05$). **Boxplot** (right) demonstrating **estradiol** (µmol/mg Cr) and **progesterone** (ng/mg Cr*10) concentrations of successful matings (**nrf1 and rQ5**) during **all test phases**. Est 1 = estradiol concentration during the first test phase, est 2 = estradiol concentration during the second test phase, est 3 = estradiol concentration during the third test phase, prog 1 = progesterone concentration during the first test phase, prog 2 = progesterone concentration during the second test phase and prog 3 = progesterone concentration during the third test phase. Significance level $p < 0.05$, * = $p < 0.001$. N = number of analysed urine samples.

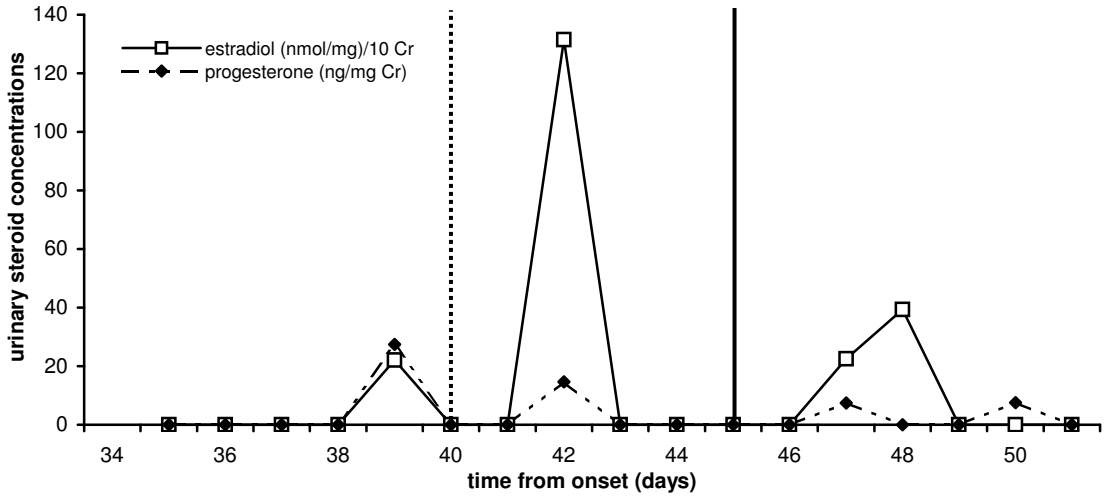


Fig. IV.2.8: Urinary estradiol and **progesterone concentrations** of **nrf3** measured during consecutive test-procedures. ····· Beginning of the second test phase, —— beginning of the third test phase.

IV.2.3.2.1 Males

Successful mating, leading to conception during the test phase three, was recorded in the males nrK1 and nrm2 (cf., tab. IV.2.2).

Two males (nrm1 and nrm2) showed a tendency towards increasing testosterone concentrations by olfactory stimulation, both with a peak on day 43 and 44, the third and fourth day of stimulation (cf., fig. IV.2.9a,b). The mean testosterone concentrations during the second test phase did, however, not significantly increase compared to the control (**nrm2**: Friedman's rank test: $\chi^2 = 1.2$, $p = 0.549$, $n = 5$, $df = 2$; Wilcoxon-rank-test: first test phase = 0.058 ± 0.041 to second test phase = 0.060 ± 0.075 nmol/mg Cr, $p = 0.892$, $Z = -0.135$; **nrm1**: Friedman's rank test: $\chi^2 = 1.0$, $p = 0.670$, $n = 2$, $df = 2$; Wilcoxon-rank-test: first test phase = 0.050 ± 0.057 to second test phase = 0.250 ± 0.199 nmol/mg Cr, $p = 0.180$, $Z = -1.342$) (appendix tab. VII.B4). It increased, on the other hand, by mating (third test phase) compared to the control (first test phase). In male nrm2 the concentration of mean testosterone increased tendentially from 0.058 ± 0.041 to 0.148 ± 0.095 nmol/mg Cr and in male nrm1 from 0.050 ± 0.057 to 0.208 ± 0.175 nmol/mg Cr (Wilcoxon-rank-test: nrm2: $p = 0.892$, $Z = -0.135$; nrm1: $p = 0.180$, $Z = -1.342$) (appendix tab. VII.B4).

The non-reproductive male **nrm5** showed an almost significant increase of the mean testosterone concentration by olfactory stimulation from 0.020 ± 0.007 to 0.048 ± 0.029 nmol/mg (Friedman's rank test: $\chi^2 = 4.6$, $p = 0.097$, $n = 3$, $df = 2$; Wilcoxon-rank-test: $p = 0.039$, $Z = -2.060$) (appendix tab. VII.B4; fig. IV.2.10). Since the following mating session resulted in decrease of the testosterone concentration, probably the Friedman's rank test did not lead to significance. This male was not interested in the female during their encounter, since it attacked his female partner after five min. The smell of that female (nrm3), however, seemingly aroused more interest in that male than the female itself.

Regarding the combined values of all non-reproductive males, a tendential increase of testosterone concentrations from 0.048 ± 0.039 nmol/mg Cr during the control to 0.067 ± 0.113 nmol/mg Cr testosterone during olfactory stimulation was recognizable (Wilcoxon-rank-test: $p = 0.482$, $Z = -0.703$) (appendix tab. VII.B4). In two cases, the testosterone concentrations decreased during olfactory stimulation, whilst in one of both (nrK1) the mean testosterone concentration increased almost significantly during the third test phase (Wilcoxon-rank-test: $p = 0.028$, $Z = -2.201$). Note, that mating of the male **nrK1** finally was successful (two pups, 101 days later). According to values of all non-reproductive males, a tendential increase of mean testosterone concentrations from 0.048 ± 0.039 nmol/mg Cr to 0.074 ± 0.078 nmol/mg Cr testosterone by mating in the

third test phase could be demonstrated (Wilcoxon-rank-test: p = 0.844 , Z = -0.196) (appendix tab. VII.B4). In three males, there was a decreasing effect of mean testosterone during the mating session. In two of them, mating was not successful, since no copulation (aggression of male nrm5) occurred. However, testosterone concentrations tendentially increased between day 47-49 (the second until the fourth day after mating) and decreased later back to the "basic level" (cf., fig. IV.2.10).

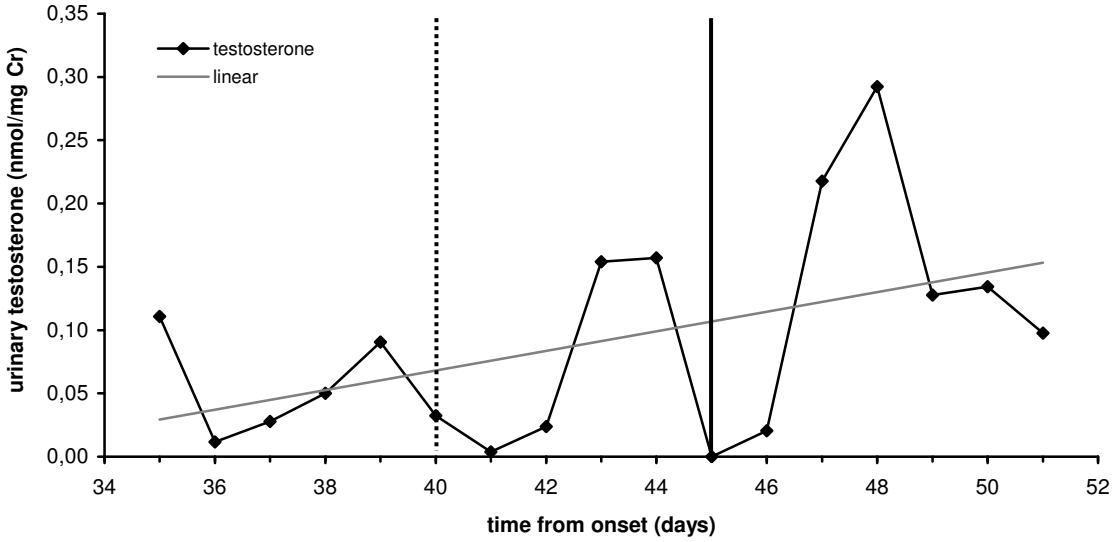


Fig. IV.2.9a: Urinary **testosterone concentrations** measured during consecutive test-procedures in **nrm2**. Test phase one without manipulation until day 39. ····· Start of the second test phase (olfactory stimulation), ——— start of the third test phase (mating).

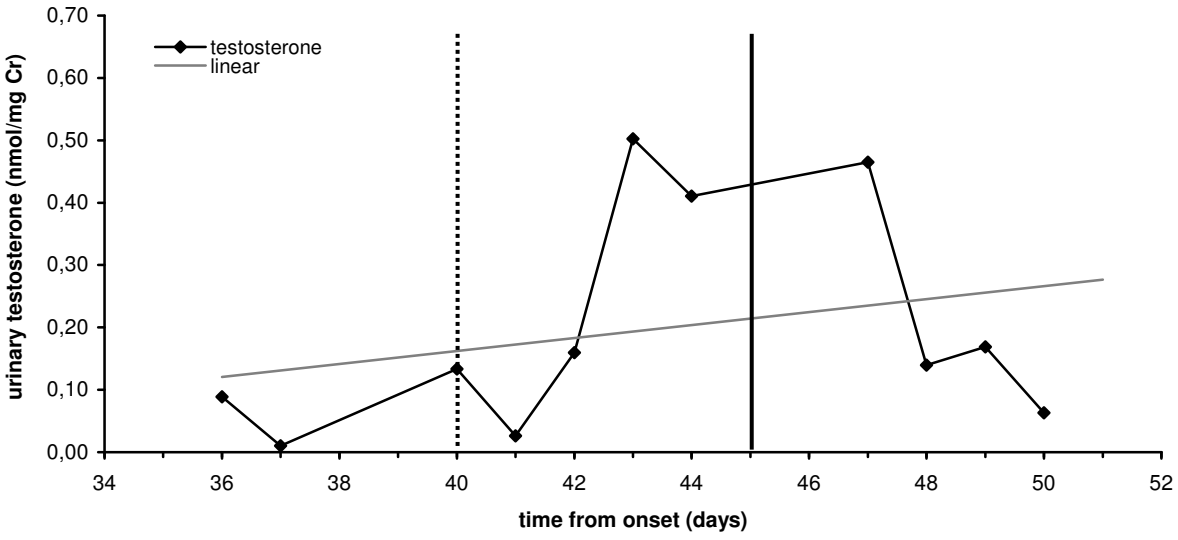


Fig. IV.2.9b: Urinary **testosterone concentrations** measured during consecutive test-procedures in **nrm1**. Test phase one without manipulation until day 39. ····· Start of the second test phase (olfactory stimulation), ——— start of the third test phase (mating).

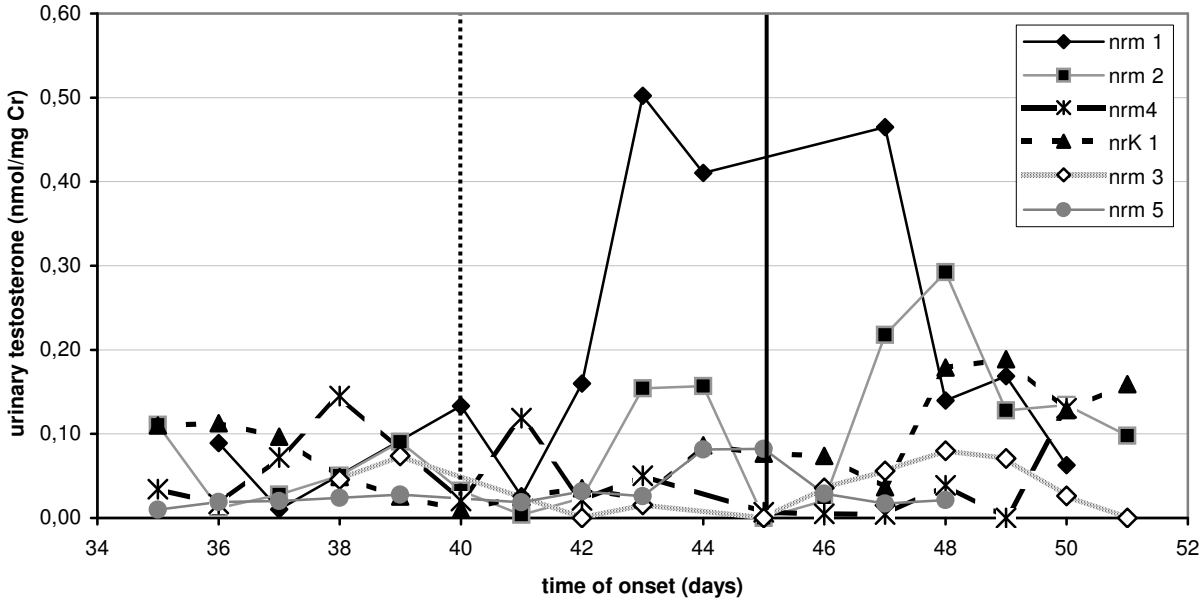


Fig. IV.2.10: Urinary **testosterone concentrations** of **all males** measured during consecutive test-procedures. Test phase one without manipulation until day 39. ····· Start of the second test phase (olfactory stimulation), _____ start of the third test phase (mating).

IV.2.3.3 Mating behaviour

When newly exposed to the odourized cage of the prospective partner during the **second test phase**, *Fukomys* mole-rats (males and females) spent considerable time sniffing the new cage.

Likewise during the **third test phase**, the mating session was accompanied by different sorts of mating behaviours, e.g. like nose to nose-contact (fig. IV.2.11a), sniffing at the flanks of the partner (fig. IV.2.11b). Moreover, a remarkable exposing of the female ano-genital area (by lifting and presenting it) was registered (fig. IV.2.11c). Nose to nose-sniffing and sniffing at the flanks was more or less marginal, whilst sniffing at the ano-genital area aroused high interest of the male, mostly accompanied by smooth tail-biting (whereas the female lifted its hind quarters almost until doing a handstand) (fig. IV.2.11d). Females, thus, seem to be more soliciting themselves and inviting than males by presenting the genital opening conspicuously. Furthermore, urination on the bottom of the test cage (fig. IV.2.11e) or (by lifting the backside and spotting) at a wall from one of the partners or defecation resulted in intensive sniffing at the marked area (fig. IV.2.11f; photo not from mating session). Strikingly, some individuals marked the wall of the test cage during the mating sessions with body secretions though pressing their flank at the wall and/or their ano-genital area on the bottom (fig. IV.2.11e, the female backmost) whilst moving forwards. Both behaviours, urination and spreading body secretion might be interpreted as marking behaviour with different sources of odour, since it provoked an intensive sniffing of the respective partners. Finally, repeated copulation up to 51-times within the first half hour (pair one, nrQ1 with nrK1) accompanied by squeaking of both partners (fig. IV.2.11h) completed mating. All those described mating behaviours were intensively prolonged over several days.

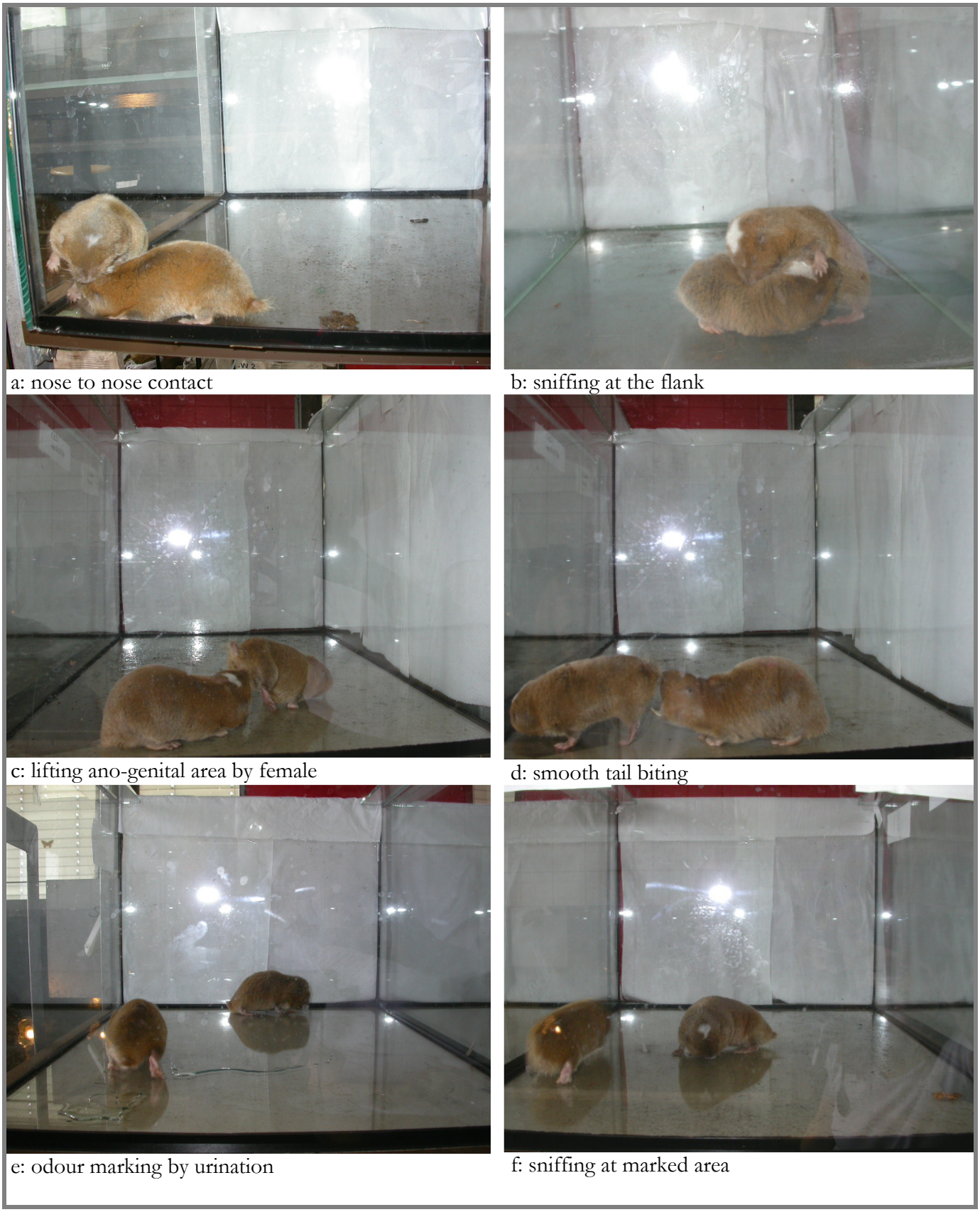
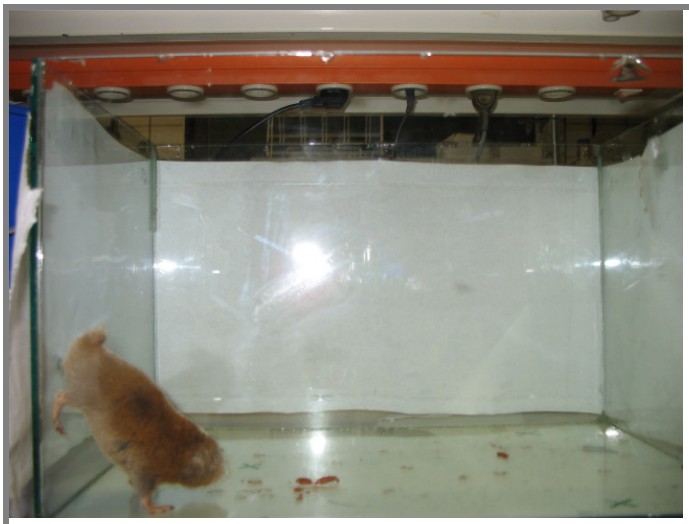


Fig. IV.2.11a-f: Demonstrating different bouts of **mating behaviour**.



g: urination at a wall

h: copulation

Fig. IV.2.11 g-h: Demonstrating different bouts of **mating behaviour**.

IV.2.4 DISCUSSION

In this chapter, a strong correlation between high mean estradiol and progesterone values and sexual activity could be demonstrated. To date, there have been no reports on estrogen concentrations in *Fukomys* mole-rats. Endocrine studies in non-breeding naked mole-rats (*Heterocephalus glaber*) revealed low urinary estrogen concentrations, confirming a lack of ovarian cyclicity and ovulation in those females. This block to ovulation was assumed to be due to inadequate concentrations of circulating LH (Faulkes et al. 1990, Westlin et al. 1994). Likewise non-breeding, sexually abstinent *Fukomys* mole-rat females do not have elevated estradiol concentrations (this study) although they exhibit all stages of follicular development (Willingstorfer et al. 1998). Although the LH-concentrations have not been studied here, in two (rQ5 and nrf3) of three successfully mated females it was recorded that olfactory stimulation tends to have an increasing effect on urinary estradiol concentrations (most probably via primary effect upon increase of LH concentrations) (cf., fig. IV.2.6b, IV.2.8). Chemosensation thus may have a preparing effect on estradiol production, which eventually, following successful mating, results in a strong estradiol increase, typical for ovulation (fig. IV.2.7a,b). The findings of this study demonstrate that the lack of repeated copulation connected with consequently successful reproduction could result in low estradiol (and progesterone – see below) concentrations, whereas the whole range of sexual activity resulted in increase of circulating estradiol concentration. This finally should explain the generally higher basal estradiol and progesterone cocentrations of reproductive active (rQ) queens.

The results of this study are partly consistent with findings of low urinary progesterone concentrations in non-reproductive females of *Fukomys damarensis* (Molteno & Bennett 2000), which all were housed together with their parents and were apparently sexually quiescent. Progesterone concentrations in non-reproductive *F. damarensis* females increased, when emancipated from their respective mother, the queens. This finding differs from the present results. In the samples of this study, progesterone was also low in non-breeding females, however, irrespective whether they were queens (nrQ) or workers (nrf). Decisive was the fact, whether they had mating opportunities (and were mated) or not. In *F. damarensis,* the queen's absence seemed to be sufficient to increase progesterone concentrations (Molteno & Bennett 2000). The authors could, however, not exclude in their study, that in the parents' absence, brothers and sisters mated with each other, a probable different explanation for the detected increasing progesterone concentrations. Apparently, in *F. anselli* and *F. kafuensis* elevated circulating progesterone in reproductively active females reflects primarily their sexual behaviour. The primary effect of sexuality

upon higher progesterone concentrations is strongly supported by findings in the copulating but non-breeding female queen, rQ5 (fig. IV.2.6b). Two progesterone peaks may be indicative for ovulation (initiating the luteal phase of the estrus) triggered by repeated copulation. It should be noted, that mating opportunity was reflected in generally higher urinary steroid concentrations in rQ5 as compared to female nrQ1 living without a male (cf., fig. IV.2.6 a,b).

It seems that sexually experienced rQ and nrQ could be primed by sexual and probably also by olfactory stimulus faster than nrf, since e.g. the female nrf3 did indeed show an estradiol peak by olfactory stimulation but not a progesterone peak (indicative for the luteal phase) (fig. IV.2.8). However, it cannot be excluded that aggressive behaviour of the first male partner suppressed the priming effect in subsequent mating in female nrf3. This could explain the strong estradiol increase during olfactory stimulation and the following decrease of that steroid the first two days of after aggressive encounter (fig. IV.2.8). This female got the olfactory stimulus of the first male (nrm5) for five days. However, about after six hours within the third test phase, the female was paired with the second male (nrm6, to date not involved in any of the test phases), because of high aggression of the first male. Soon after the first encounter between the female and the second partner (nrm6) it showed the whole repertoire of mating behaviour (cf., above, chapter IV.2.1.3.3). The female demonstrated and exposed the vagina and both sniffed intensively at each other. They copulated the first time after 15 min, probably with intromission accompanied by typical squeaking of the female. After half an hour they copulated four times. The first vaginal smear of this female was easily to obtain, probably because it copulated before. Lots of sperms and granulocytes, both stages 6, could be detected. Stillbirth occurred after 108 days.

Monitoring the cortisol concentration could help to interpret the influence of stress on circulating estradiol and progesterone. Obviously, the second male aroused sexual interest, probably also responsible for the estradiol increase on day 53 and 54, three and four days after start of the mating session. Moreover, testing the circulating estradiol and progesterone concentrations a certain period of time (e.g. half year) after the first successful reproduction should confirm the finding of generally higher estradiol and progesterone concentrations in reproductively active females. This would lead to affirmative insight to differentiate reproductive queens from non-reproductive females on the basis on circulating steroids. Nevertheless, even without this second confirmation, obviously queens can methodologically be distinguished from their female offspring by circulating urinary estradiol- and progesterone concentrations. This easily applicable

method is thus apparently appropriate for husbandry purposes and field check of wild-captured *Fukomys* mole-rats.

The present results are so far consistent with previous findings and suggestions by Willingstorfer et al. (1998) and Burda (1999). Assessment of LH cocentrations in females with different sexual experience and activity are needed to complement our insight.

Since nucleated cells are the major cell population in the smear in mice and rats at proestrus and develop into cornified cells (Nelson et al 1982, Maeda et al. 2000), one purpose was to find a periodical correlation between occurrence and frequency of nucleated and cornified cells. However, cornified cells were not found to be a reliable indicator in mole-rats. This cell type was always represented and constituted never the major cell population in smears, probably due to mechanical abrasion of the vaginal epithel. Due to lack of ovarian cycle and spontaneous ovulation, colpocytology showed no cyclic change of cellular composition (except weakly, in reproductively active queens, rQ; tab. IV.2.3, fig. IV.2.2; appendix fig. VII.B4). A cytological picture of an estrus was never detected, neither in reproductively active queens nor in not-reproductive females. Even after successful mating, no correlation between induced ovulation and the frequency of typical estrus-like cells (increase of cornified cells, decrease of nucleated cells and decrease of leucocytes in late proestrus and estrus) could be detected. This, however, may explain that reproductive queens do not differ from non-reproductive females on the basis of colpocytological investigation in spite of large differences of circulating basal steroid concentrations. Due to this fact also no correlation between cycling urinary steroids (estradiol and progesterone concentration) and the vaginal cell composition (nucleated or cornified cells) was detectable. If thus neither estradiol nor progesterone do influence the uterine lining, the uterus seems to be permanently ready for embryonal implantation in queens as well as in "workers". This is consistent with the finding of high proportions of nucleated cells found in almost all vaginal smears as well (fig. IV.2.3, appendix fig. VII.B 4-7). The results of Willingstorfer et al. (1999) confirm this hypothesis by findings of highly developed ovarian follicles. Histological investigations of the uterus of *Fukomys* mole-rats would complete colpocyctological findings and interpretations of the study here. Anatomical and histological studies in non-breeding females of the naked mole-rats (*Heterocephalus glaber*) found, in contrast, thin walled and narrow uteri and mainly primordial and primary follicles in the ovary, contrary to breeding females, suggesting functionally quiescent and prepubescent reproductive morphology (Kayanja & Jarvis 1971). Obviously the ovarian cyclicity of naked mole-rat queens and the absence of it in non-breeders are reflected by histological findings of the uterus and ovaries, contrary to ovarian findings in *Fukomys* mole-rats. The assumed general morphological readiness of

sexually inexperienced *Fukomys* females makes sense due to a potential dispersal from the own family, in spite of the fact that they need to be primed longer than sexual experienced queens (see above). It is moreover consistent with the aseasonality of breeding.

Referring to the methodological point of view, in sum, colpocytology is not an applicable method to diagnose estrus in the studied *Fukomys* mole-rat species.

Kramer (2006) found no differences in generally higher testosterone concentrations of reproductive kings compared to non-reproductive males. This finding, besides studies on sperm motility and testicular morphometrics corresponds to the respective findings in the Highveld mole-rats (van Rensburg et al. 2003) and to studies on testosterone concentrations of the Damaraland mole-rats (Bennett 1994), leading the authors to the conclusion that non-reproductive males are physiologically not suppressed in contrast to females of both species. Olfactory stimulation with the odour of unfamiliar females probably influences the endocrine system of males (this study), recognizable by slight increase of urinary testosterone concentrations. A short increase of testosterone concentrations was detected, partly accompanied by successful mating (including animals interest on behavioural level, repeated copulation and eventual conception), though reaching the "basal level" some days later (fig. IV.2.10). This "backwards effect" of short testosterone increase corresponds to findings of Kramer (2006). In this study, two to four days after mating session (day 47-50), the testosterone concentrations of most male subjects decreased to their initial concentrations (cf., fig. IV.2.10). Otherwise, the repeated copulation and reproduction of the reproductive pair should theoretically have resulted in generally higher testosterone concentrations of kings, which was not the case. Anyhow, it should be kept in mind, that the lower testing ranges of testosterone are error-prone (Kramer 2006), which should lead to careful interpretations made here, referring to the priming role of olfaction and mating on urinary testosterone concentrations in males. To date, there are no further data available regarding the priming effect of olfactory signals and/or mating on the testosterone concentrations in reproductive and non-reproductive males in different mole-rats species.

In summary, the present study shows a marked impact of sexual activity on female urinary steroid concentrations. Although olfactory stimulation of females appears to increase the estradiol concentrations, it is not sufficient to reach high estradiol concentrations registered following successful mating. This study supports conclusions of previous studies (Burda 1999, Willingstorfer et al. 1999) that *Fukomys* mole-rats are induced ovulators, primed exclusively through repeated and regular sexual activity and not solely by a single copulation or absence from the queen. Prolonged olfactory stimulation could lead to stronger effects.

IV.3 SEMIOCHEMICALS - HS-SPME-GC-MS-ANALYSIS

IV.3.1 INTRODUCTION

In rodents, chemical communication is mediated mainly by urinary signals. Scent communication conveys a wide range of chemical information about the owner like species, sex, individual identity, kinship, social status (i.e. dominance), and the reproductive status. In this way, scent marking includes the ability to recognize individuals (e.g. Hurst & Beynon 2004). In social animals, chemical communication is expected to be especially complex since the maintenance of appropriate social interactions largely depends on the ability of group or family members to distinguish between one another and to respond differentially (Fanjul et al. 2003).

In the eusocial *Fukomys* mole-rats, individual odour discrimination was demonstrated for ano-genital odours within their families (Heth et al. 2002). It was demonstrated that *Fukomys* mole-rats also use odours in other contexts: e.g. *F. anselli* and *F. kafuensis* non-reproductive males discriminate and prefer ano-genital odours of breeding females to those of non-breeding females (cf., chapter IV.1.4.2). Moreover, this trend was also found when using urine as an odour source, however with lower response of the males (this study). Heth and his colleagues (2004) in addition found, that *F. anselli* males preferred ano-genital odours of unrelated females to those of their unfamiliar (= forgotten) sisters and also preferred to mate with unrelated females rather than with own forgotten sisters. Thus, in this genus besides individual identity, odours in ano-genital secretions and/or urine most probably are important for behavioural responses in many further contexts, e.g. sex, individual and/or food preferences, and reproductive status (breeding queen or non-breeding female, king or non-reproductive male). It is not yet clear, however, to which extent urine is involved in these chemical communication processes underground. Chemical constituents of urine (and other odour sources) thus may vary between reproductive and social categories and also according to particular background situations (e.g. diet) in which communication takes place.

To date, no chemical-analytical investigation was done using urine or other potential odour sources in *Fukomys* mole-rats. Menzies and his colleagues (1992) found, however, in a subterranean but phylogenically not related and solitary living blind mole-rat (*Spalax ehrenbergi*) pheromonal activity in lipids of urine, extracted by the solvent dimethylchloride. Unfortunately they probably loosed and/or destroyed relevant urinary compounds during the extraction process. A new analytical approach, head-space solid-phase microextraction coupled with gas chromatography and mass spectrometry

(HS-SPME-GC-MS), was developed in the current study to overcome such analytical problems. The main purpose was to elucidate differences in the volatile urinary profile between different families, individuals, sex and reproductive status (king, queen, daughter or son), as well as to identify at least partially compounds (classes), constituting those urinary patterns. Moreover, the impact of food preference on urinary metabolites was investigated on the example of carrot and potato diets respectively.

This new, solvent-free method is based on the use of a syringe-mounted fused-silica rod (fibre) coated with an absorptive organic phase, e.g. polydimethylsiloxane (PDMS), or an adsorptive one, e.g. carboxene (CAR) or carbowax (CW). The fibre absorbs and/or adsorbs analytes in the head space from water soluble sample like urine. After this extraction process, the analytes can be thermally desorbed into the heated GC injection port and analysed directly by coupled MS. The main advantage of SPME is the complete elimination of organic solvents. This method, moreover, reduces the amount of samples needed to run an analysis, sometimes from millilitres to microlitres. Analytical conditions are thus improved when using biological samples from small animals (Auger et al. 1998, Baltussen et al. 2002, Kayali-Sayadi et al. 2003). Kayali-Syadi and his colleagues (2003) found the CAR/PDMS fibre to deliver best results of trapping target pheromones in the house mouse (*M. m. domesticus*). Since target compounds, probably odours and/or pheromones, in *Fukomys* mole-rats are hitherto unknown, the search for relevant compounds was more or less blind. At most, well known lipophilic ligand MUP-compounds (major urinary protein-compounds) of a model species, the house mouse (*M. m. domesticus*), served as possible target substances and were under the first focus: e.g. the terpenes like α- and β-farnesene, geraniol, or pheromone active compounds like 2-*sec*-dihydrothiazole or 3,4-dehydro-*exo*-brevicomin (Novotny et al. 1990 and 1999b, Hurst et al. 1998, Zhang et al. 2007). But also different terpenoids and other compound classes, like alcohols, aldehydes or ketones, esters, lactoles, or even fatty acids could not be excluded to be of potential pheromonal relevance in *Fukomys*, since most of those substances have been reported in different species (Rasmussen & Perrin 1999, Soini et al. 2005b, Zhang et al. 2005, Zhang et al. 2008). In contrast to the mouse urine, in *Fukomys* mole-rats however, the chromatogram and mass spectra were sharpest when using a three-component 50/30 µl DVB/CAR™/PDMS™Stable Flex™-fibre (50 µm divenyl benzene/30 µm carboxene/polydimethyl siloxane-fibre).

In the current study, therefore, the urinary profiles of six different families kept on or without potato and carrot diet were chemically analysed with this new approach. In addition, 16 individuals of three families of both sex and all reproductive states, i.e. queens, kings and their non-reproductive offspring, were under focus to find out more about the modifications

of the urinary constituents and their olfactory meaning to the animals in the above mentioned different animal categories and test conditions.

IV.3.2 MATERIAL AND METHODS

IV.3.2.1 Animals

To focus on differences and/or similarities between urinary volatiles, six laboratory families of closely related *Fukomys* species were studied; three families of *F. anselli* and three families of hybrids (*F. anselli* x *F. kafuensis*). The urine of all families was measured twice (except for family five, tab. IV.3.1, see below). The composition of the family urine is described in more detail below (cf., chapter IV.3.1.2.3).

To test the impact of diet on family urinary volatiles, the described procedure was performed in animals kept on a carrot diet and a potato diet respectively with family two.

Urinary volatiles of individuals, different sex and reproductive status were tested in 16 individuals of three different families (family one, two and four). In each family, at least four individuals of different categories (Q, K, nrf and nrm) were analysed (tab. IV.3.2).

Tab. IV.3.1: *Fukomys* families examined for urinary family odours. FA = *F. anselli*, FKA = hybrids of *F. anselli* and *F. kafuensis*.

family code	species	family	first measurement	second measurement
1	Anselli	FA13	x	x
2	Anselli	FA12	x	x
3	Anselli	FA4	x	x
4	Hybrid	FKA1	x	x
5	Hybrid	FKA2	x	
6	Hybrid	FKA8	x	x

Tab. IV.3.2: Individuals examined for urinary individual odours of different sex and reproductive status (rQ = reproductive queen, K = king, nrf = non-reproductive female/daughter, nrm = non-reproductive male/son). FA = *F. anselli*, FKA = hybrids of *F.anselli* and *F. kafuensis*.

species/ family	family code	individual code	individual	reproductive status
F.anselli/ FA13	1	A	914	rQ
		B	668	nrf
		C	688	K
		D	061	nrm
		E	900	nrm
		F	07.2	nrf
hybrids (*F.a.* x *F.k.*)/ FKA1	4	A	511	rQ
		B	485	K
		C	659	nrf
		D	903	nrm
F.anselli/ FA12	2	A	023	rQ
		B	754	K
		C	854	nrm
		D	087	nrf
		E	013	nrf
		F	287	nrf

IV.3.2.2 Samples

Since analytical GC-MS investigations are highly sensitive to impurities, the sample preparation was always performed with analytical clean glass and metal laboratory equipment, e.g. tweezers or head-space vials. After each application, it was cleaned three times with acetone, three times with ethyl acetate and three times with ethanol by rinsing with the respective pure (p.a., *per analysis*) solvent. To avoid extra GC-peaks, ethanol was used in the end, since the internal standard (see below) was solved in ethanol as well. Following the solvent cleaning, the laboratory equipment was heated for at least 1 hour at 250° C, then coated with fresh aluminium foil and stored until use in a cleaned exhaust hood. All sample preparation was performed in an exhaust hood which was lined with fresh aluminium foil. The solvents were stored in a cleaned and heated 100 ml glass vial covered with Teflon screw cap which was stable to heating at a maximum of 180° C (Schott AG, Mainz, Germany). To reduce further handling contamination nitrile gloves were used.

IV.3.2.3 Urine sample preparation

Collecting urine was performed as described in chapter IV.1.2.1.2. For monitoring family odours, urine of at least four different individuals from one family, the king, the queen, one son, and one daughter, were mixed to a total volume of 1,800 µl. If possible, urine of up to of six individuals from one family was pooled together (cf., tab. IV.3.1 and IV.3.2, above).

The ratio of urine between all individuals was kept balanced according to the sex and urine amount, i.e. in case of four individuals the amount of 450 µl of each individual were mixed and in the case of six individuals 300 µl urine of each. If the amount of fresh collected urine was not enough, it was supplemented with frozen urine. Samples were mixed with Eppendorf tubes in a 5 ml injection-vial (38 x 22 mm; Scherf Präzision Europa GmbH, Meiningen, Germany) and a 2 mm cleaned and heated PTFE-magnet stir bar (a heat stabile stir bar of Teflon®, chemical = polytetrafluorethene) was supplemented into the injection vial. Teflon® coated crimp Caps (N 20 TS/HS; Macherey-Nagel, Düren, Germany) were manually fixed on the head-space vials with a hand cramper for 20 mm seals.

IV.3.2.4 Optimizing the experimental condition for SPME

Volatile compounds above each urine pool were sampled using a SPME (solid-phase microextraction) fibre and immediately analysed using GC-MS. Different parameters had to be varied in order to generate useful GC-peaks, e.g. the accumulation time and temperature of equilibration and a suitable SPME-fibre (DVB/CAR™/PDMS™Stable Flex™-fibre = 50 µm divenyl benzene/30 µm carboxene/polydimethyl siloxane-fibre, PDMS™-fibre or the CAR™/PDMS™-fibre from Supelco Corp., Bellanfonte, PA, USA) according to the polarity or non-polarity of the target compounds. Moreover, the immersion and headspace methods were tested (see below).

The DVB/CAR™/PDMS™Stable Flex™-fibre SPME-fibre was finally conditioned for 1 hour at 250° C in the GC injection port before each extraction, since this fibre was found to be the best one (cf., the results of chapter IV.3.1.3.1). New SPME fibres were conditioned at 265° C for 1 hour before use. No salt addition or pH adjustment was performed on the *Fukomys* mole-rat urine. To keep the temperature during head space-adsorbing constant, the 5 ml head-space vial containing 1,800 µl urine was mounted on a magnetic stirrer (Heidolph Instruments GmbH & Co. KG, Schwalbach, Germany) submerged in a water bath of 80° C and was then equilibrated for 1 hour. The water bath was stirred constantly with magnetic stir bar at a speed of 1,250 rpm (fig. IV.3.1). The fibre was in close proximity to the urine (or ddH_2O as a blank), i.e. head-space, but direct contact to urine was always avoided. Directly after extraction procedure, the SPME-fibre was injected into the GC-column (see below).

Fig. IV.3.1: Head-space sampling (HS) of urine for **SPME-CG-MS** (solid-phase microextraction gas chromatography and mass spectrometry) analysis, showing the sampling of dd H_2O as a blank.

IV.3.2.5 Gas chromatography – mass spectrometry (GC-MS)

Chromatographic separations were performed using a Hewlett-Packard (Palo Alto, CA, USA) HP 5890 II instrument with a split-splitless injector equipped with a special liner (0.75 mm I.D.; Supelco, Bellafonte, PA, USA) adapted to SPME. Moreover, a DB 35 (35% phenyl-methyl-polydimethyl siloxane) column (30m x 0.25mm I.D., with a 0.25 µm film) and a Hewlett Packard mass spectrometer (MS, HP 5970 Series Mass Selective Detector) as detector were used. A leak-proof seal between inlet liner and the injection port body were achieved by Therm-O-Ring Seals ¼ (Supelco, Bellafonte, PA, USA).

The loaded SPME-fibre was injected directly into the GC-column. The injector temperature was set to 250° C and the splitless injection was opened after 2 min The initial GC oven temperature (35° C) was held for 15 min, ramped to 60° C at 3° C min^{-1} and held for 5 min and then ramped to 150° C at 10° C min^{-1} and held for 1 min and finally ramped to 290° C at 25° C min^{-1} and kept at the end temperature of 290° C for further 10 min Helium was used as a carrier gas with a column flow rate of 1.0 ml min^{-1}. The solvent delay was set to 2.6 min.

A blank was always run in the GC to ensure that no contamination was left on the fibre. A further blank of the GC-column was run additionally only in the case, when the origin of the contamination had to be checked, i.e. if it originated from the SPME-fibre or from the GC-column. Contamination was always eliminated through heating, before a new GC of a urine sample was measured. Graphs were visualized by ORIGIN®7-software (OriginLab, Northampton, USA).

Internal standards (IS)

Aromatical compounds like terpenes, aldehydes or ketones, e.g. fluorobenzaldehyde, 4-pentenal, 1-(S)-α-pinene, diterpene-mix, vertinpropionate (2-methoxy-3-isopropylpyrazine) and methylenanthate, were tested to find out the best internal standard, of which vertinpropionate and 1-(S)-α-pinene emerged to operate best. From 1-(S)-α-pinene, 1 µl with a dilution of 1:1,000 was added to 1,800 µl urine samples. The dilution of vertinpropionate was 1:10,000, of which 1 µl was added to the urine sample as well. By this procedure every urine sample of family odours was measured four times, i.e. twice with and twice without internal standard to avoid any possible interference, overlap or peaks of possible break down products of standards to the urinary profile.

Calibration

Both internal standards were solved in ethanol. From 1-(S)-α-pinene a 1: 1 000 diluted stock solution was prepared by solving 100 µl 1-(S)-α-pinene in 90,900 µl EtOH. A dilution series (1:10,000; 1:5,000 and 1:1,000) was prepared from that stock solution. Moreover, a series of 1:1,000; 1:2,000 and 1:20,000 dilution of vertinpropionate was solved in ethanol. From these stock solutions 1 µl of each was added respectively to 1,800 µl urine samples (or 1,800 µl ddH_2O as standard blank for testing appropriate internal standard, see above) and prepared for the SPME equilibration (cf., chapter IV.3.1.4.2). Afterwards it was injected into the GC.

Peak detection

To compare different profiles, peaks were matched by their retention time and mass spectra. Repetitive peaks of different chromatograms were searched and the contribution of each peak to the overall area of the whole urinary volatile profile (except the solvent delay) was calculated in percent (%-area) and thus used for a quantitative analysis. This allowed the comparison of the urinary volatile peak profiles quantitatively between different families or between different individuals according to the proportions of every relevant peak to those profiles. When a peak was matched by its retention time and its mass spectra while the detection limit was to low to calculate the area, its %-area was set to 0.001% to enable further statistical calculation. Peaks, which were not included into the mean %-area for the confidence interval calculation (cf., next page), did not get a peak number (cf., tab. IV.3.3).

Compounds were identified preliminarily from their mass spectra by the NIST mass spectral reference library (National Institutes of Standards and Technology, 2002). Peak areas were calibrated (cf., above) with 1-(S)-α-pinene and vertinpropionate.

The compound ß-caryophyllene was used with a dilution of 1:10,000 to validate the retention time of peak 23 (see below) and for confirmation of the compound additionally to the NIST library. The amount of 1 µl ß-caryophyllene was used with a dilution of 1:10,000 (in EtOH) in 1,800 µl ddH_2O. The preparation procedure of HS-SPMC-GC-MS was according to the procedure of calibration standards (see above).

Samples of every family were run fourth (twice with and twice without internal standards), except of family five because of lack of urine. The mean of two measures (without internal standards) were used for calculation of %-area for each peak. From family two, the urine samples were run twice under "normal" food intake. Furthermore, the samples were collected and measured after one week of potato diet. After a break of one week with normal diet, this procedure was repeated with a carrot diet. The urine samples of potato and carrot diets could only be measured once with- and once without internal standards, because of lack of urine. Samples of individuals were run twice (once with and once without internal standards).

To compare profiles between individuals, sexes and reproductive status (Q, K, nrf, nrm), the peak areas (PAs) were normalized by dividing a respective PA by the PA of internal standard (IS), i.e. PA x 100/IS. Vertinpropionate was used as the reference IS. Due to striking differences of concentrations of urinary components in the TIC (total ion chromatogram) between the above mentioned animal categories, certain peaks were chosen for this continuative quantitative analysis: The peaks 33, 36, 37, and 40 were checked for differences between Q and nrf while peaks 23 and 24 were checked for differences between males and females in general (cf., chapter IV.3.1.3).

IV.3.2.6 Statistics and quantitative analytical data

Because of the limited amount of urine of animals kept either on carrot or potato diet, one HS-SPME-GC-MS measure of each condition and did not allow to prove the significance statistically, i.e. to compare the concentrations of altered "diet-peaks" with normal peaks.

Differences in concentrations of certain peaks (cf., above) between sexes (males and females) and reproductive status of a queen or a king in comparison to their non-reproductive daughters and sons were performed using the non-parametric Mann-Whitney *U*-test, respectively. The significance was accepted at $p < 0.05$. One nrf (individual E) of family two was excluded from statistical data, since outliers were assumed.

IV.3.3 RESULTS

IV.3.3.1 SPME equilibration

The chromatogram and mass spectra were sharpest when using a three-component 50/30 µm DVB/CAR™/PDMS™Stable Flex™-fibre (50 µm divenyl benzene/30 µm carboxene/polydimethyl siloxane-fibre Supelco Corp., Bellafonte, PA, USA). Neither the PDMS-fibre for non-polar high molecular weight compounds nor the CAR/PDMS-fibre was successful in the present study. The method of immersion was always ineffective. For the head-space transfer the best temperature was 80° C for 1 hour (appendix tab. VII.C1).

IV.3.3.2 Gas chromatography – mass spectrometry

All fibre blanks were absolutely free of dirt (appendix fig. VII.C1). Using the 50/30 µm DVB/CAR™/PDMS™Stable Flex™-fibre, the terpene 1-(S)-α-pinene and vertinpropionate (2-methoxy-3-isopropylpyrazine) were found to be appropriate internal standards, since they provided useful reproducible peaks, with high linearity of the regression curve (vertinpropionate, R^2 = 0.995; 1-(S)-α-pinene, R^2 = 0.999; appendix fig. VII.C2 and VII.C3), reliable retention time (RT vertinpropionate = 33.4 min, RT 1-(S)-α-pinene = 15.2 min; appendix fig. VII.C4 and VII.C5), appropriate match of mass spectra (appendix fig. VII.C6a,b), and one manageable interference with the profile of family and individual urinary samples (the masking of peak at RT = 33.4 min by vertinpropionate, see below; appendix fig. VII.C7a-c). The calibration of 1-(S)-α-pinene and vertinpropionate thus shows that the retention times and integrated areas are reproducible values, making both to reliable parameters.

Since vertinpropionate had a peak in the range of relevant urinary mole-rats' peaks and also sharper peaks with a smaller peak width compared to 1-(S)-α-pinene, it was used as the reference IS. However, since the peak of vertinpropionate masked the component at RT = 33.4 min (1-methyl-4-(1-methylethenyl)-benzene, match 883 in mass spectrum of the family two; cf., appendix fig. VII.C7a-c)), it was necessary to measure every urinary sample of families four times to handle those interferences (cf., method of IS).

Quantification of peaks (PA/IS see below) was therefore performed with vertinpropionate. The spectral identification and confirmation of vertinpropionate using the mass spectrum (MS) demonstrate a very high match (935) to the reference and high probability (92.5%) of this compound by using the NIST-library 2002 (appendix fig. VII.C6a,b). Because of constant RT's between 33.3-33.4 min of repeated measures and the same mass spectrum in the urinary samples compared to the dd H_2O blank, vertinpropionate proved to be an appropriate and reliable IS.

IV.3.3.3 Differences/similarities between families and during diet

Many of the urinary peaks could not be identified or were only tentatively assessed from the mass spectra. Thus further analytical effort is necessary for that purpose.

Altogether 41 different peaks were found, which were present in all six family samples (tab. IV.3.3, below). Except for family three, the GC-pattern was very similar (fig. IV.3.2a-c, fig. IV.3.3). The largest peaks in all families were peaks 6, 7, 19 and 20. Peak 6 is extraordinary high in family three with 36,5 %-area (explaining the large error bar representing confidence interval, $\alpha = 0.05$; fig. IV.3.4) and peak 39 is present only in this family (cf., tab. IV.3.2).

Component at RT = 33.9 min could not be integrated separately from peak 33.6/7 because of overlap. Component at RT = 34.6 min was present in family two only during a potato diet and was missing in every other sample. Peaks 28-30 at RT = 41.1-41.4 min were missing in some families of hybrids and were similarly very large during a carrot diet in family two (cf., tab. IV.3.3, fig. IV.3.4 and fig. IV.3.5a). Component at RT = 43.6 min was missing in family one, two and six and could not be calculated to the %-area. Peaks 31, 32 and 36 were conspiciously higher during a carrot diet (fig. IV.3.4). The carrot diet shifted and altered the urinary volatile profile, so that six peaks (9, 10, 14, 16, 17, 18) disappeared and further six peaks (25, 28, 29, 30, 31, 36) got significantly higher %-areas (fig. IV.3.5a).

During the potato diet, seven peaks (9, 11, 14, 22, 31, 32, 35) disappeared and two further peaks (13 and 26) rose significantly in their overall %-area contribution.

The potato and the carrot diet thus definitely shifted the urinary peak profiles, proved by two measures of each food condition (with and without internal standards) resulting in large similarity of repeated measures of each food condition (cf., fig. 3.5.c,d; appendix fig. VII.C8 and VII.C9).

Tab. IV.3.3: List of peaks (41 components) found in urine of **different families** of *Fukomys* mole-rats during **normal food intake**, **potato** and **carrot diet**, using the method of HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry). In grey, peaks of compounds present in every family. Bold text indicates identified compounds by mass spectra. RT = retention time (min), **cp** = "carrot peak", **pp** = "potato peak".

peak	RT [min]	Compound	%-area (mean) normal n=6	%-area potato diet n=1	%-area carrot diet n=1	peak missing in family
1	28.7	?	0.934	1.425	2.418	
2	29.3	benzaldehyde?	2.087	1.563	0.001	
3	32.3	**4-nonanone**	0.963	0.001	0,001	3
4	32.7	**(E)-2-octenal**	1.739	0.612	0.863	
5	33.3	1-methyl-4(1-methylethenyl)-benzene?	1.497	3.545	1.745	

peak	RT [min]	Compound	%-area (mean) normal n=6	%-area potato diet n=1	%-area carrot diet n=1	peak missing in family
6	33.6/7	**overlap of 1,3,8-p-mentatriene and 4-methylphenol** (affected by **potato and carrot diet)**	12.202	1.373	4.385	
7	34.3/4	**acetophenone**	5.404	5.401	1.225	
8	35.5	(E)-nonenal?	1.859	1.666	1.970	
9	35.9	?	1.417		/	
10	36.0	?	2.769	0.878	/	
11	37.0/1	?	1.512	/	0.622	
12	37.2	4-undecanone? behind siloxane peak	2.567	1.469	1.955	
13	37.9	1-phenyl-2-butanone?	2.153	**5.190**	2.039	
14	38.0/1	?	0.229	/	/	
15	38.5	α,4-dimethyl -3-cyclohexene-1-acetaldehyde,?	1.177	0.935	0.967	
16	38.6	6-methyl-2-pyridinecarbaldehyde?	0.364	0.553	/	
17	38.7	2-none-4-one?	0.630	2,149	/	
18	39.4	siloxane	1.319	0.001	0.001	
19	39.5	**2-methoxy-4-vinylphenol**	5.478	3.910	1.179	
20	39.7	**ß-caryophyllene**	6.660	2.707	4.609	
21	39.9 **pp**	1-(3,6,6-trimethyl-1,6,7,7α-tetrahydrocyclopenta[c]pyran-1-yl)ethanone ?	1.811	1.043	1.628	
22	40.0	1-(2-aminophenyl)-ethanone?	1.221		/	
23	40.2/3	α-caryophyllene?	1.476	0.655	1.144	
24	40.4	hexanedioic acid, bis(1-methylethyl) ester?	1.365	1.151	1.346	
25	40.6 **cp**	1-(1,5-dimethyl-4-hexenyl)-4-methyl-benzene?	1.389	1.449	**15.309**	
26	40.7	1-{2-[3-methyl-3-(5-methyl-furan-2-yl)-butyl]-oxiran}-2-yl?	2.901	**5.520**	0.841	
27	40.9/41.0	a siloxane	2.832	0.001	0.940	
28	41.1 **cp** and **pp**	2,4-bis(1,1-dimethylethyl)-phenol?	0.903	2.055	**5.545**	4/5
29	41.2 **cp**	similar to quinoline, 4,8-dimethyl-quinoline?	1.318	1.742	**6.894**	6
30	41.4 **cp**	α-vatirenene?	0.601	2.027	**3.505**	4/5/6
31	41.6 **cp**	?	1.023	/	**8.122**	
32	41.7 **cp**	6,6-dimethyl-2-vinylidenebicyclo[3.1.1]heptane?	0.995	/	**5.188**	
33	41.9	6,10-dimethyl-3,5,9-undecatrien-2-one?	1.177	2.496	0.865	
34	42.0	benzoic acid?, 2,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester and/or ß-caryophyllene oxide?	2.681	1.366	2.551	
35	42.1	?	1.195	/	3.111	
36	42.2	β-vatirenene? E-farnesene epoxide?	1.712	2.913	**5.715**	
37	43.3/4	**1-hexadecanol**	1.381	0.627	0.863	
	43.6	2-methyl-5-(1,2,2-trimethylcyclopentyl)-(S)-phenol?				1/2/6
38	43.9	?	1.007	0.563	0.451	
39	44.3	1,1,5-pentadecanediol?	1.266			only in family 3
40	44.7	1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester?	1.216	1.226	0.604	
41	45.7	**octadecanoic acidbutyl ester?**	1.511	1.402	0.547	

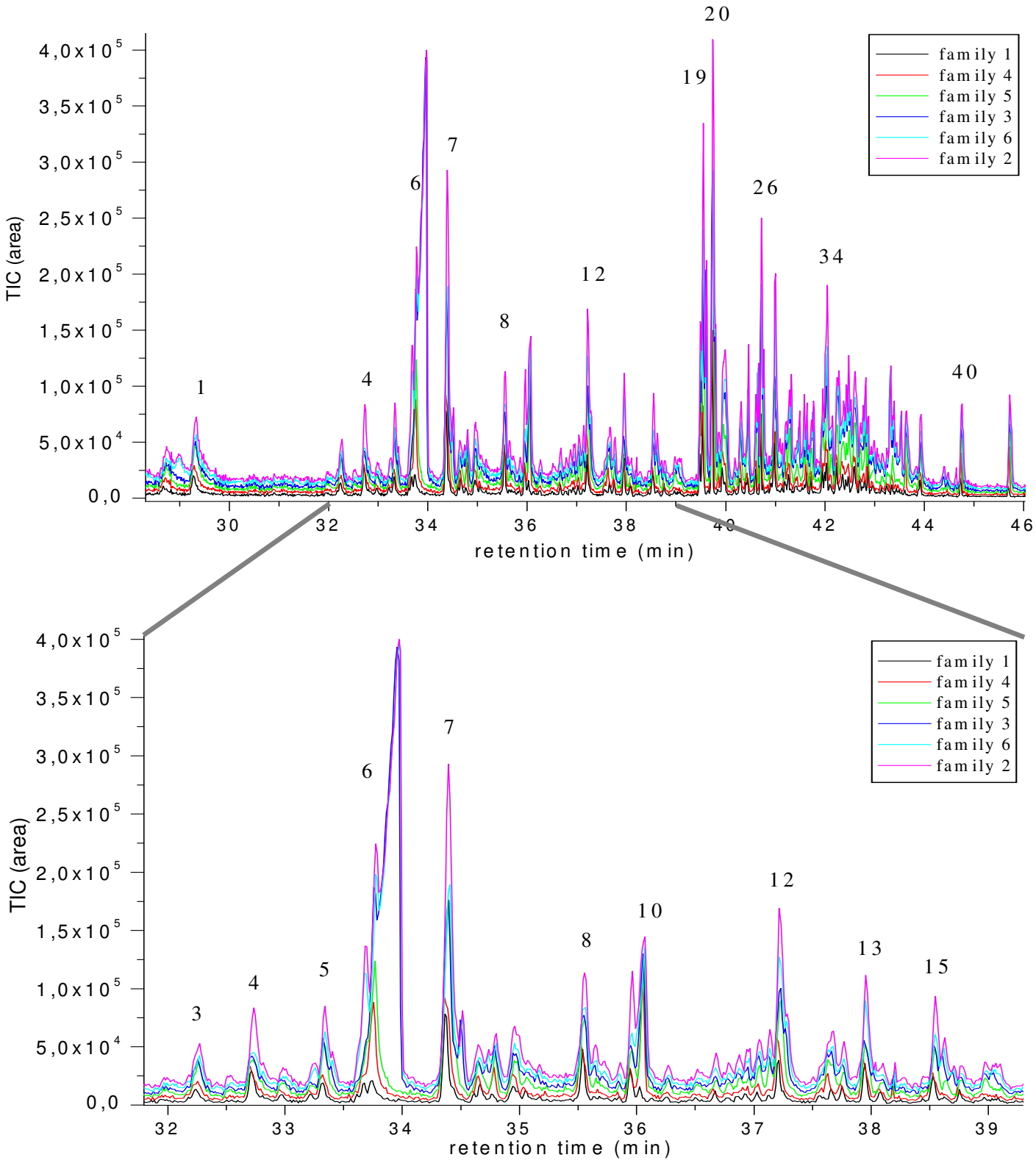


Fig. IV.3.2a,b: TICs (total ion chromatograms) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of urine samples from **5 families** of Ansell's and hybrid *Fukomys* mole-rats illustrating similarity. Graphs are stacked in layers avoiding overlap. Below the detailed chromatograms of the RT-range between **RT = 32 – 39 min**. Several peaks are labelled with numbers. RT = retention time (min).

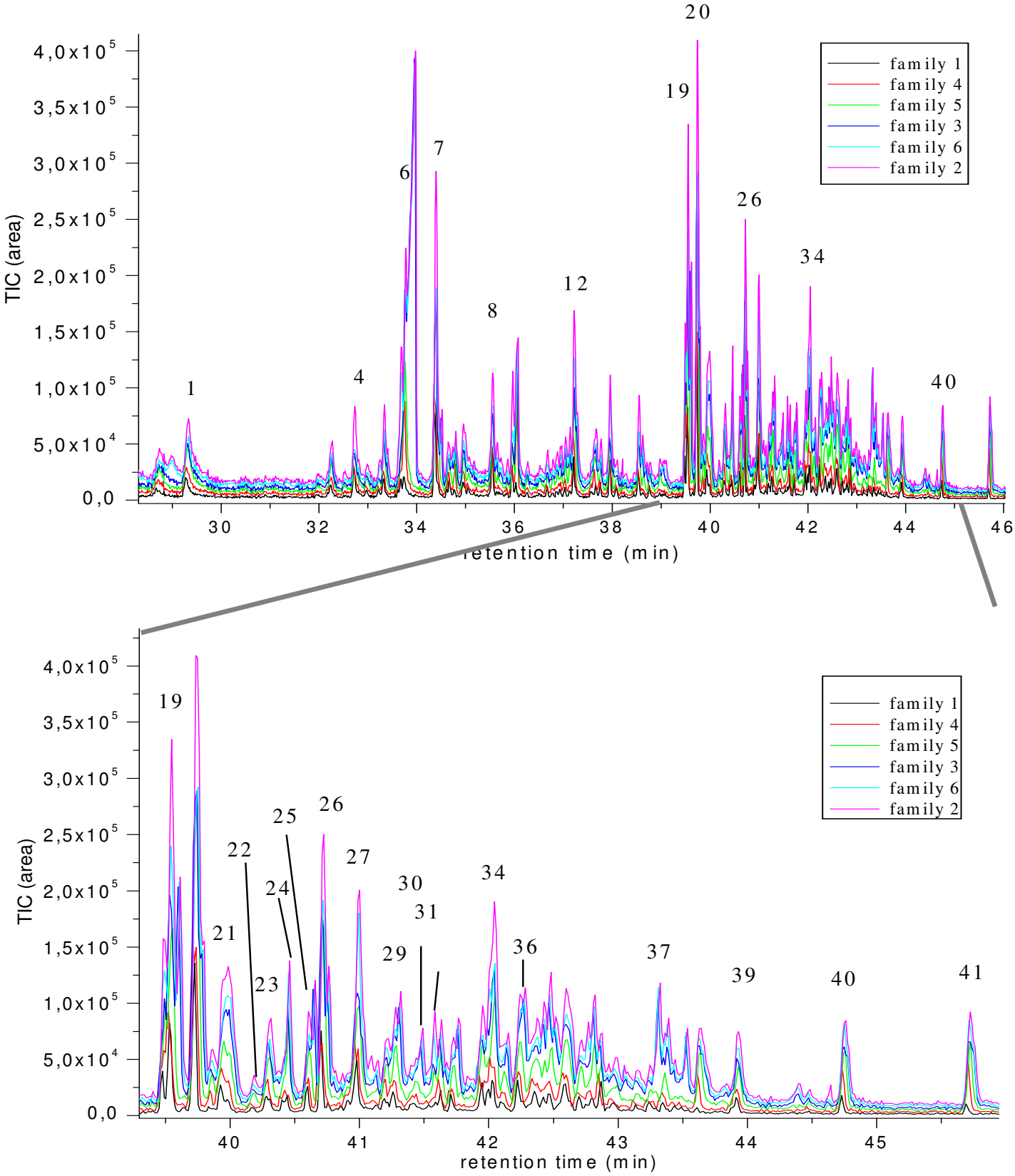


Fig. IV.3.2a,c: TICs (total ion chromatograms) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of urine samples from **5 families** of Ansell's and hybrid *Fukomys* mole-rats illustrating similarity. Graphs are stacked in layers avoiding overlap. Below the detailed chromatograms of the RT-range between **RT = 39 – 45 min**. Several peaks are labelled with numbers. RT = retention time (min).

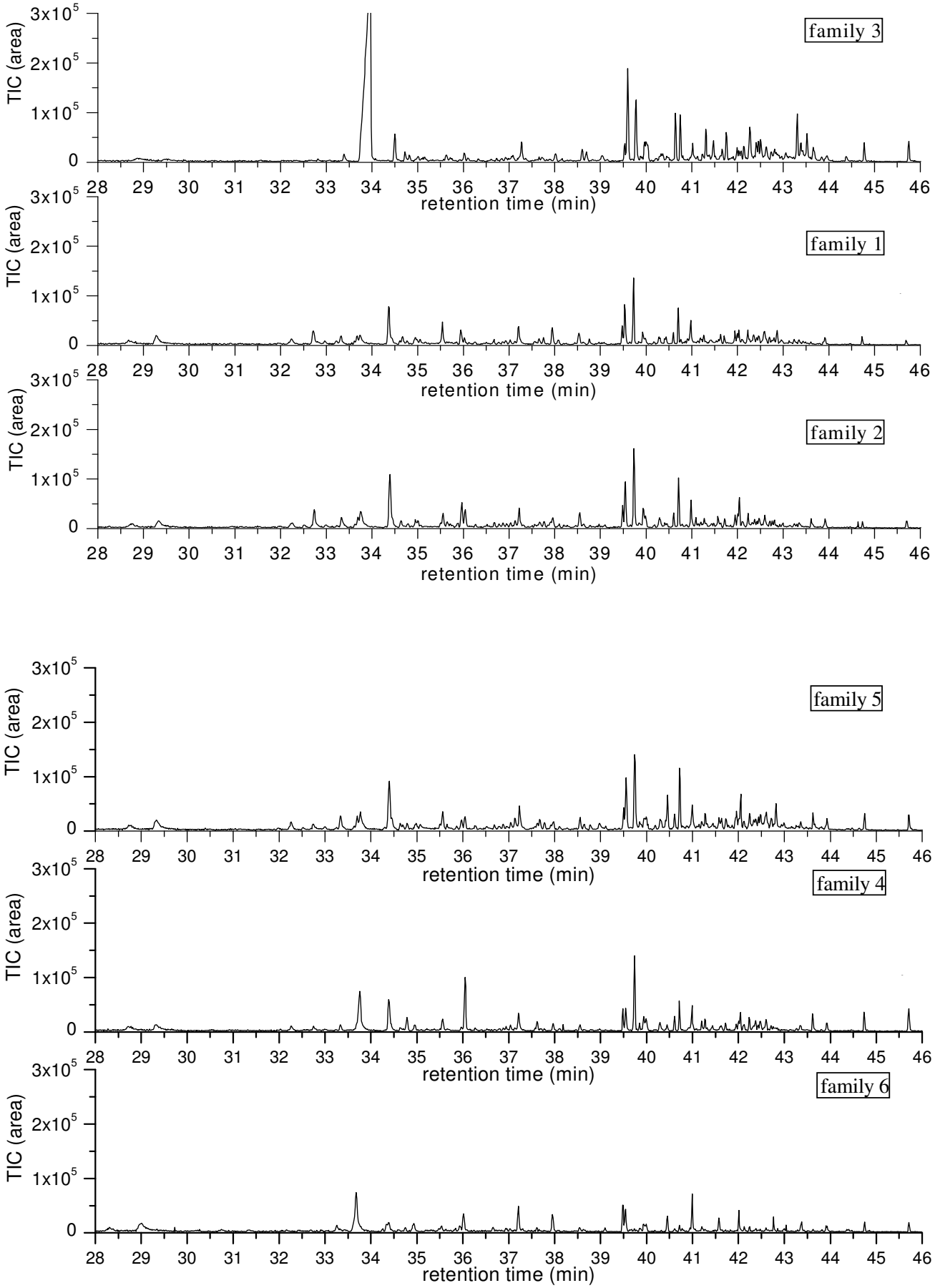


Fig. IV.3.3: TICs (total ion chromatograms) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of urine samples from **family 1-6** of Ansell's and hybrid *Fukomys* mole-rats **without diet** illustrating similarity between family peak profiles.

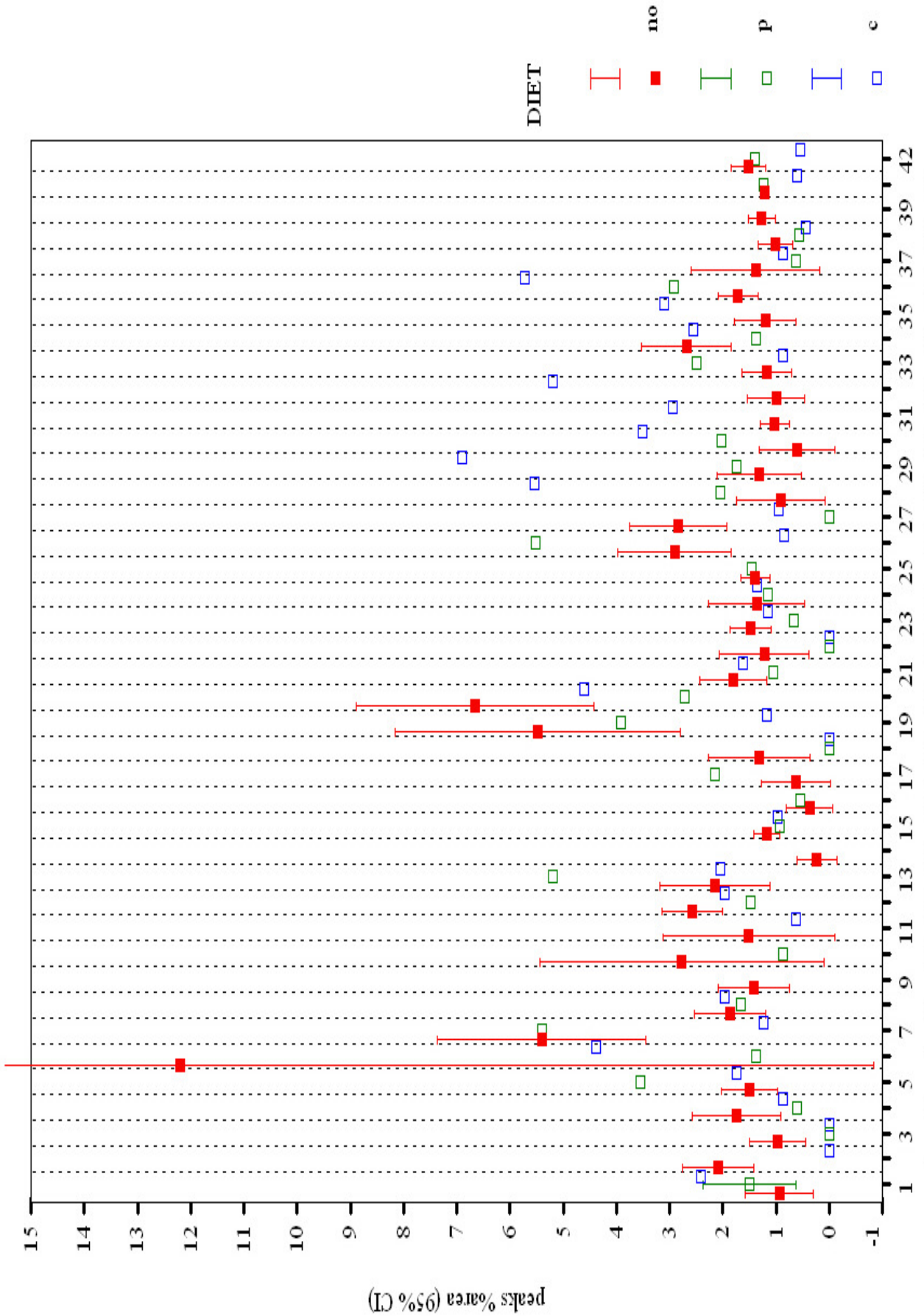


Fig. IV.3.4.: Comparison of **%-area** of peaks of **all tested families**. Red squares represent mean of %-area during **normal food intake** with error bars (with 95% lower and upper confidence interval, CI; $\alpha = 0.05$) compared to %-area of peaks from one measure during **potato diet** and one measure during **carrot diet**. p = potato diet, c = carrot diet. On x-axis are the numbers of peaks.

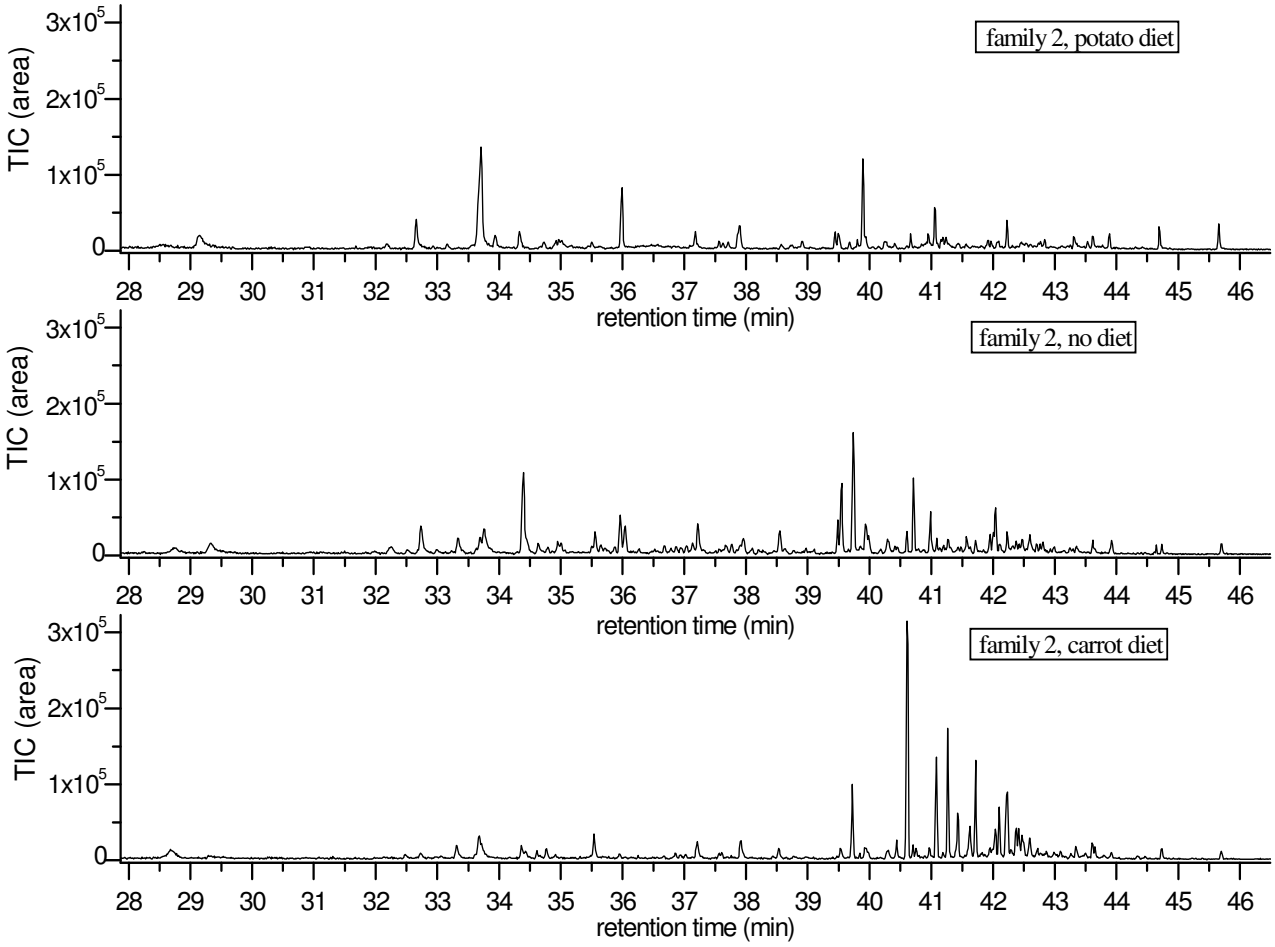


Fig. IV.3.5a: TICs (total ion chromatograms) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of urine samples from family two of Ansell's *Fukomys* mole-rats **without diet**, with **carrot** and **potato diet** illustrating **differences** in the peak profiles.

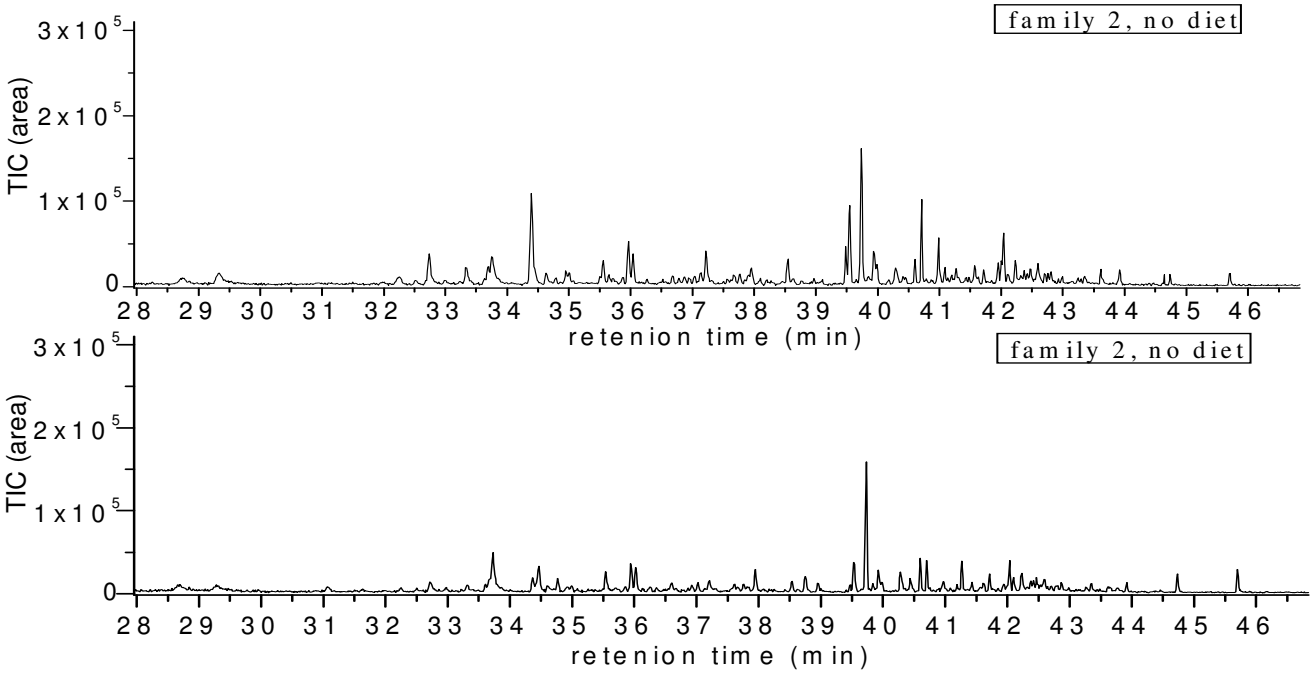


Fig. IV.3.5b: TICs (total ion chromatogram) of two measures obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from family two of an Ansell's *Fukomys* mole-rats without diet illustrating **similarity of repeated measures**.

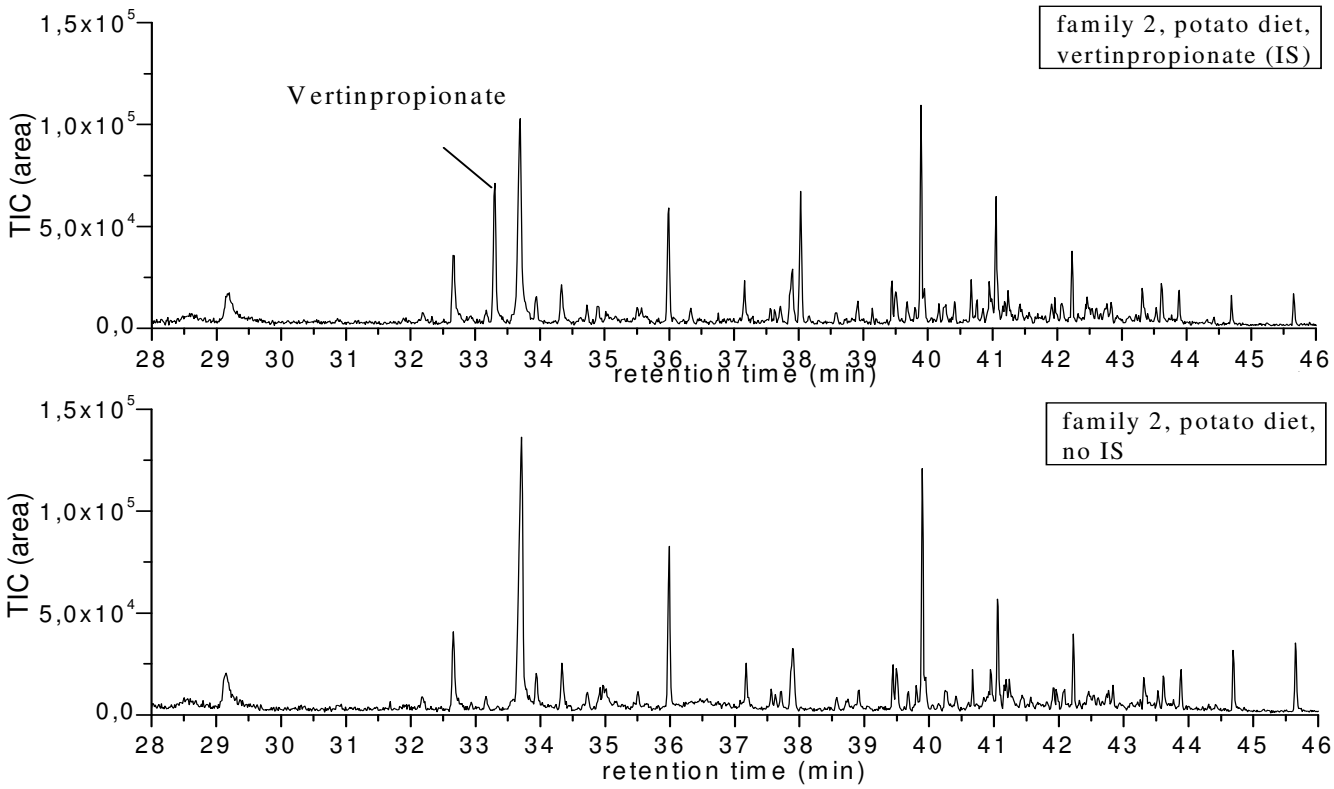


Fig. IV.3.5c: TICs (total ion chromatograms) of two measures obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from family two of Ansell's *Fukomys* mole-rats during potato diet illustrating **similarity of repeated measures**.

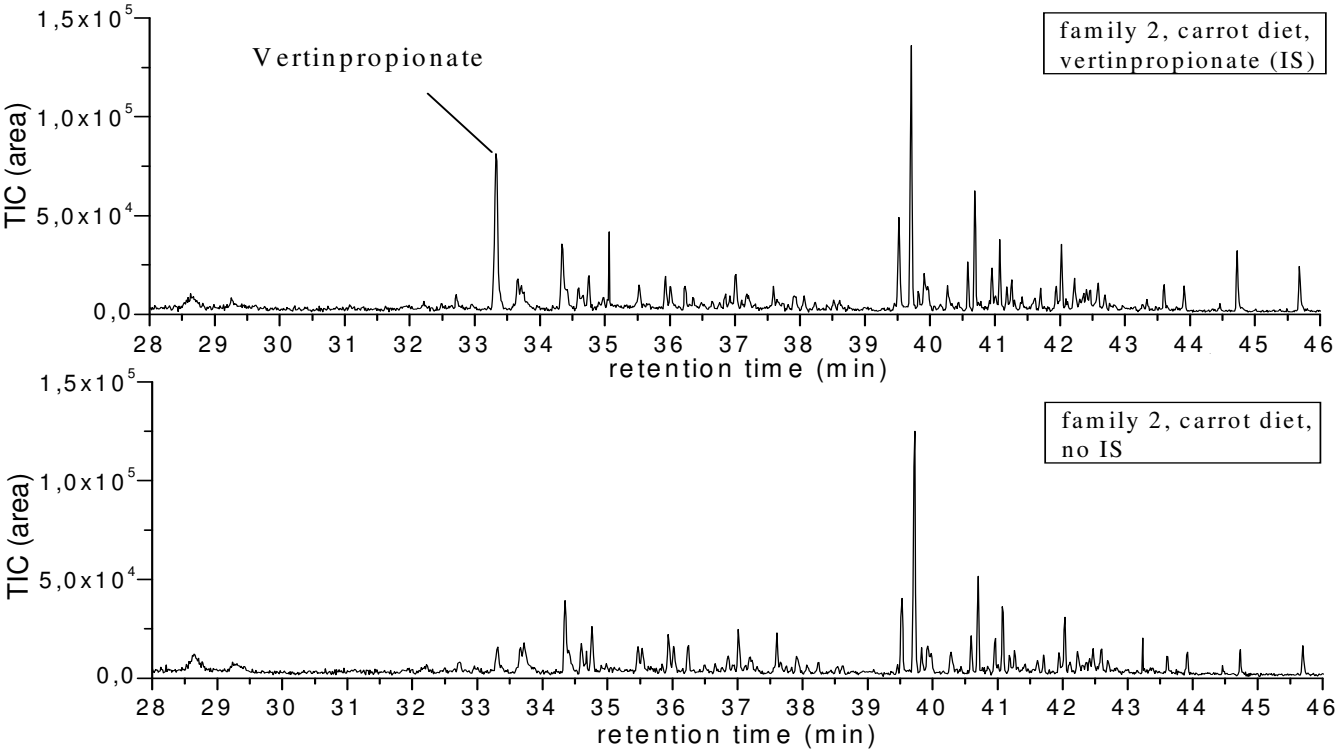


Fig. IV.3.5d: TICs (total ion chromatograms) of two measures obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from family two of Ansell's *Fukomys* mole-rats during carrot diet illustrating **similarity of repeated measures**.

IV.3.3.4 Differences between individuals

Individuals of three different families were under focus (cf., tab. IV.3.2). Compared to urinary profiles of families, additional peaks were found when searching for individuality: Peak 5, 6, 8, 12.2, 15, 21, 38.2, 41-46, 48 (tab. IV.3.4, below).

Inter-individual variations were found according to the sex and reproductive status (Q or nrf) and not on individuality per se, since the urinary peak profiles within males and within females were similar, contrary to their reproductive status (cf., chapter IV.3.1.2.4, fig. IV.3.6). According to sex and reproductive status, the chemical profile of individuals consisted up to 51 peaks (tab. IV.3.4, below).

Tab. IV.3.4: List of peaks (51 components) found in the **urine of individuals** belonging to different reproductive categories; the queen (Q), the king (K), sons (nrm) and daughters (nrf) from *Fukomys* mole-rats, using the method of **HS-SPME-GC-MS** (head-space solid-phase microextraction gas chromatography and mass spectrometry). In grey peaks of compounds, newly detected in individual chromatograms of different sexual categories. Red capitals indicate gender specific compounds. Bold text indicates identified compounds by mass spectra. RT = retention time (min); f = female; x = presence of a peak; **-** = absence of a peak, cp = "carrot peak", pp = "potato peak".

family peaks	indi-vidual peaks	RT [min]	Compound	Q n=3	K n=3	nrf n=6	nrm n=5	sexual peak category
1	1	28.7	?	x	x	x	x	
2	2	29.3	benzaldehyde?	x	x	x	x	
3	3	32.3	**4-nonanone**	x	-	-	-	Q-peak
4	4	32.7	**E-2-octenal**	x	x	x	x	
-	5	32.9	1-octen-3-one?	x	-	x	-	f-peak
-	6	33.2	4-methyl-2-hexanone?	x	-	x	1 nrm	f-peak
5	6.2	33.3	1-methyl-4(1-methylethenyl)-benzene?	x	x	x	x	
6	7	33.6	**1,3,8-p-mentatriene**	x	x	x	x	
-	8	33.7	**4-methylphenol**	x	x	x	x	high values in individuals of FKA 1
7	9	34.3/4	**acetophenone**	x	x	x	x	
8	10	35.5	(E)-nonenal?	x	x	x	x	
9	11	35.9	2-isopropenyl-5-methylhex-4-enal?	-	x	x	x	not the Q
10	12	36.0	benzylmethylketone?	x	x	x	x	
-	12.2	36.2	α,α 4-trimethyl-3-cyclohexene-1-methanol?	-	x	2 nrf	x	not the Q
11	13	37.0/1	?	-	x	x	x	not the Q
12	14	37.2	4-undecanone? behind siloxane peak	x	x	x	x	
-	15	37.7	2-undecanone?	x	-	-	x	
13	16	37.9	1-phenyl-2-butanone?	x	x	x	1 nrm	
14	17	38.0/1	2-methyl-5-(1-methylethenyl)-2-cyclohexen-1-one?	-	1 K	x	x	
15	18	38.5	α,4-dimethyl-3-cyclohexene-1-acetaldehyde?	x	x	x	x	
16	19	38.6	6-methyl-2-pyridinecarbaldehyde?	x	x	x	x	
17	20	38.7	2-none-4-one?	x	-	x	x	

family peaks	indi-vidual peaks	RT [min]	Compound	Q n=3	K n=3	nrf n=6	nrm n=5	sexual peak category
-	21	38.9	?	x	-	x	x	
18	22	39.4	siloxane	x	x	x	x	
19	23	39.5	2-methoxy-4-venylphenol	x	x	x	x	
20	24	39.7	**ß-caryophyllene**	x	**x**	x	**x**	male peak
21	25	39.9 cp	1-(3,6,6-trimethyl-1,6,7,7-α -tetrahydrocyclopenta[c]pyran-1-yl)ethanone?	x	x	x	x	
22	26	40.0	1-(2-aminophenyl)-ethanone,?or 5-acetyl-2-methylpyridine?	x	x	x	x	
23	27	40.2/3	α-caryophyllene?	x	x	2 nrf	x	
24	28	40.4	hexanedioic acid, bis(1-methylethyl) ester?	-	1 K	x	1 nrf	nrf-peak?
25	29	40.6 cp	1-(1,5-dimethyl-4-hexenyl)-4-methyl-benzene?	x	x	x	x	
26	30	40.7	1-{2-[3-methyl-3-(5-methyl-furan-2-yl)-butyl]-oxiran}-2-yl?	x	x	x	x	
27	31	40.9/4	a siloxane	x	x	x	x	
28	32	41.1 cp and pp	2,4-bis(1,1-dimethylethyl)-phenol?,	x	x	x	x	
29	33	41.2 cp	similiar to quinoline?, 4,8-dimethyl-quinoline?	x	x	x	x	
30	34	41.4 cp	α-vatirenene?	x	x	x	x	
31	35	41.6 cp	?	x	x	x	x	
32	36	41.7 cp	6,6-dimethyl-2-vinylidenebicyclo[3.1.1]heptane?	x	x	x	x	
33	37	41.9	6,10-dimethyl-3,5,9-undecatrien-2-one?	x	x	x	x	
34	38	42.01	benzoic acid, 2,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester ?	x	1K	x	x	
-	38.2	42.05	ß-caryophyllene oxide?	x	x	-	x	not nrf
35	39	42.1	?	x	x	x	x	
36	40	42.2	beta-vatirenene? E-farnesene epoxide?	x	-	x	1 nrm	f-paek
-	41	42.3	farnesene epoxide?	x	x	x	x	
-	42	42.4	3,3,5-trimethyl-2-(3-methylphenyl)-2-hexanol?	x	x	x	2 nrm	
-	43	42.6	2-furancarboxylic acid, decyl ester?	x	x	x	x	
-	44	42.7	2-(1-methylpropyl)- cyclopentanone?	x	-	x	-	3 nrm with different MS-pattern
-	45	42.8	1,4-cyclododecanedione?	x	-	x	-	f-paek?
-	46	42.9	ç dodecalactone?	2 Q	-	2 nrf	-	f-paek?
37	47	43.3/4	**1-hexadecanol**	-	-	x	-	nrf-peak
-	48	43.6	2-methyl-5-(1,2,2-trimethylcyclopentyl)-(S)-phenol?	x	x	x	x	
38	49	43.9	?	x	x	x	x	
39	-	44.3	1,1,5-pentadecanediol?					
40	50	44.7	**hexadecanoic acid butylester**	x	x	x	x	
41	51	45.7	**octadecanoic acid butyl ester**	x	x	x	x	

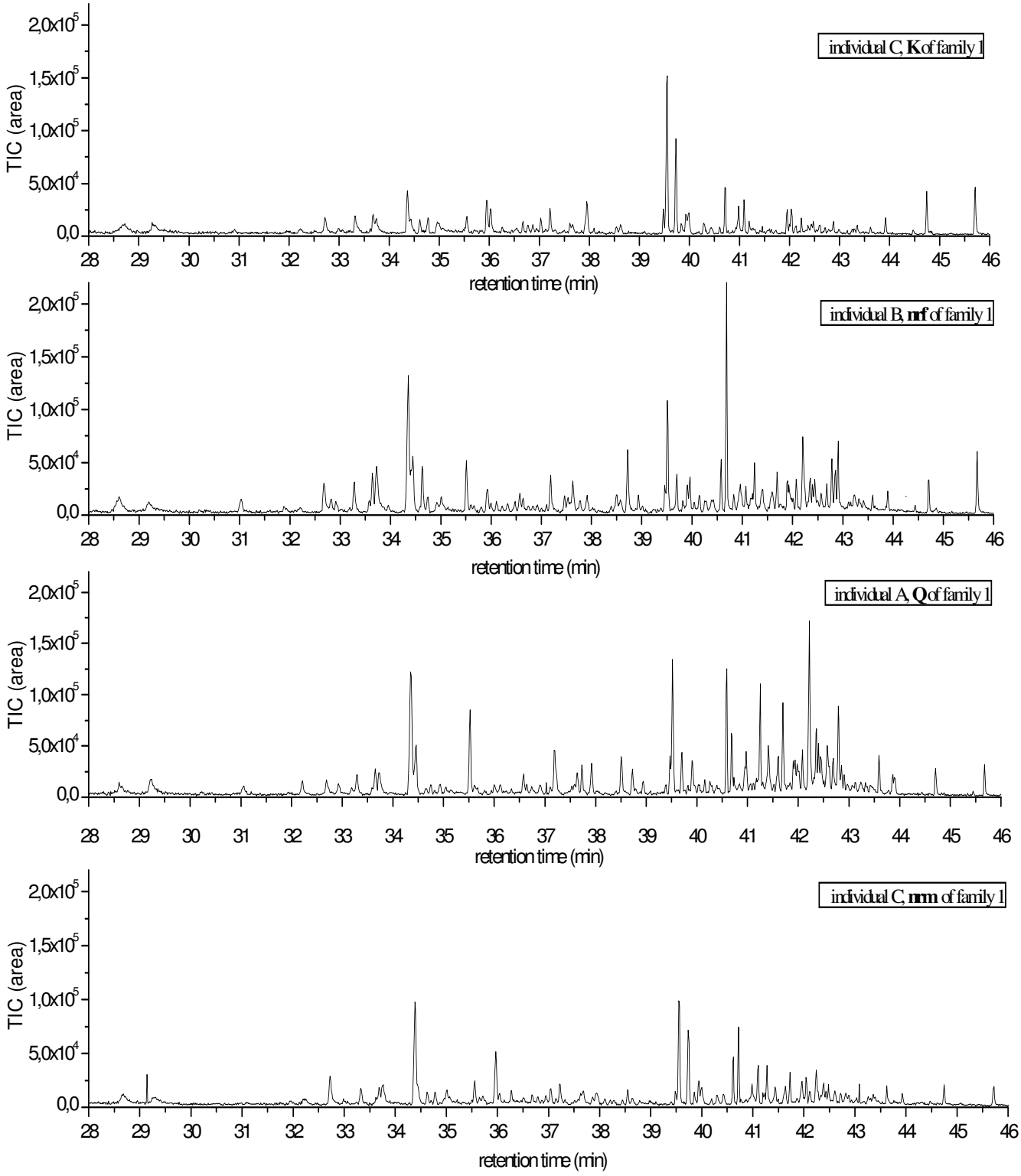


Fig. IV.3.6: TICs (total ion chromatograms) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of urine samples from four different individuals of family one of Ansell's *Fukomys* mole-rats demonstrating differences between males (K and nrm) compared to females of **different reproductive status** (Q and nrf). The relative similarities within females and within males accentuate **differences between sex.**

IV.3.3.5 Differences between queens and non-reproductive females

Altogether 54 different components were found, of which 39 were present in all the samples (cf., tab. IV.3.4). One queen-specific peak (peak 3 at RT = 32.3 min, identified as 4-nonanone) was found, since it could be proved in all Q and was absent in all nrf, K and nrm (cf., tab. IV.3.4, fig. IV.3.7). Furthermore, one component was absent in queens (peak 17 at RT = 38.0 min) whilst it was present in all the nrf (except one and not integrated in two nrf), in one K and three nrm individuals. Surprisingly, this component vanished during the carrot- and during potato diet respectively. One peak, number 33 at RT = 41.7 min, which increased during carrot diet, was significantly higher in Q than in nrfs (Mann-Whitney *U*-test, n = 8, p = 0.036, U = 0.0, mean Q = 69.7 (PA/IS), mean nrf = 37.5 (PA/IS); fig. IV.3.7 and IV.3.8, tab. IV.3.4). Three further compounds were not significant but conspicuously higher in Q than in nrfs: Peak 36 at RT = 41.7 min which vanished during potato diet (Mann-Whitney *U*-test, n = 8, p = 0.071, U = 1.0, mean Q = 54.6 (PA/IS), mean nrf = 24.6 (PA/IS)) and peak 37 at RT = 41.9 min (Mann-Whitney *U*-test, n = 8, p = 0.571, U = 5.0, mean Q = 56.8 (PA/IS), mean nrf = 42.5 (PA/IS)). That peak was not affected during carrot or potato diet. Peak 40 at RT = 42.2 min was high in Q, low in nrfs, absent in K and present in low amount (%-area) in one nrm (Mann-Whitney *U*-test, n = 8, p = 0.250, U = 3.0, mean Q = 92.8 (PA/IS), mean nrf = 41.5 (PA/IS) (tab. IV.3.4 and IV.3.5; fig. IV.3.7 and IV.3.8)). With mass spectra, the component 33 was identified as a quinoline and component 40 as β-vatirenene or E-farnesene epoxide, but further analytical work need to be carried out to confirm this finding. Peaks 36 and 37 could not be identified by mass spectra yet.

Comparing the TICs (total ion chromatograms) of non-reproductive females did not reveal any individual variation, within families. However, compared to queens, their respective daughters generally had lower peak %-areas in the range of RT = 39 min till RT = 43 min. This pattern was found in all the three families (fig. IV.3.9a-c), except for one daughter (nrf E) of family two, whose urinary profile resembled that of the queens, but it did not have the queen-specific component 4-nonanone at RT = 32.3 min (fig. IV.3.9d). Furthermore, peak 17 at RT = 38.0 min was present, which normally was absent in queens (see above).

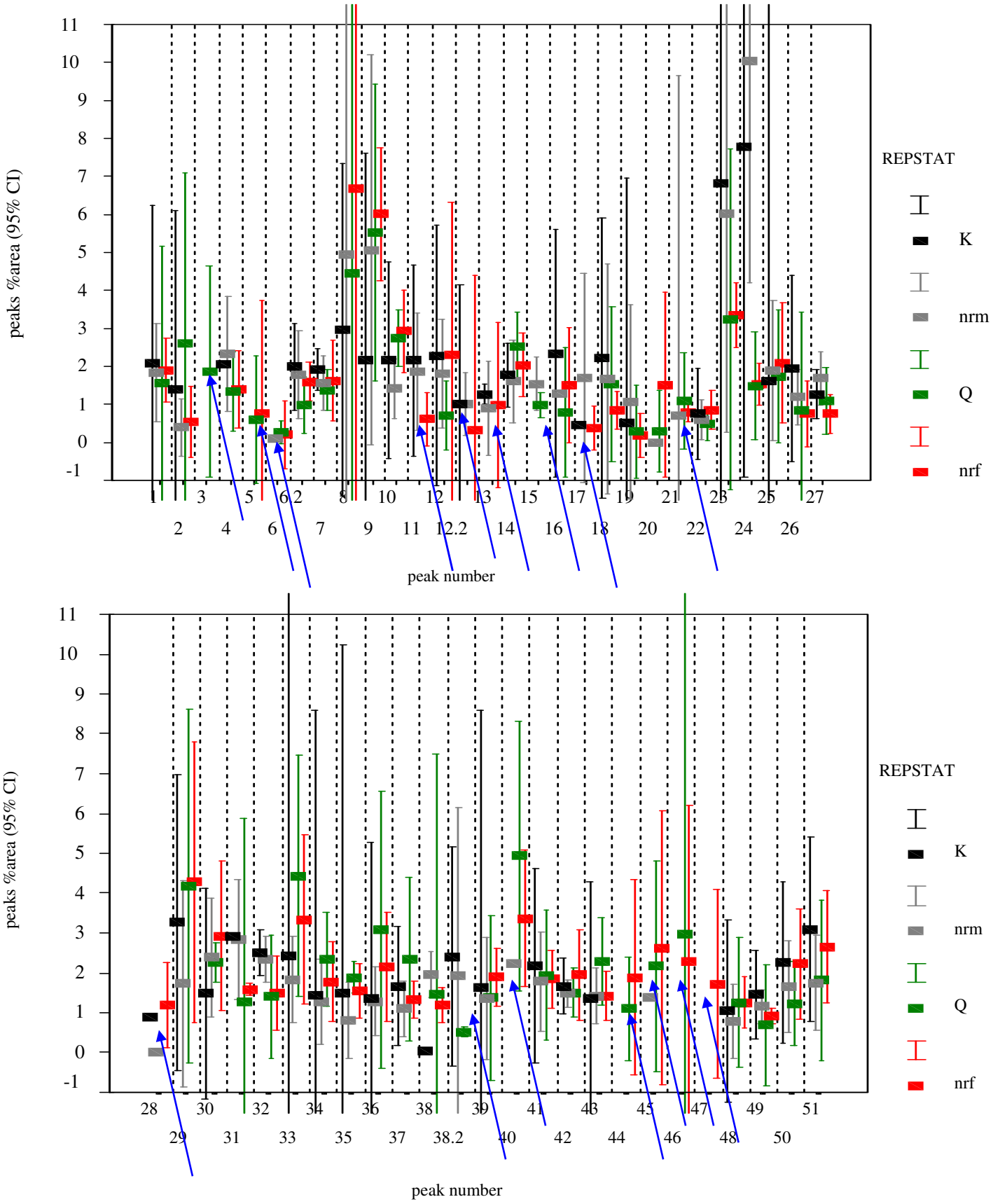


Fig. IV.3.7: Comparison of %-area of peaks 1-27, above and peaks 28-51, below between **kings** (K), **non-reproductive males** (nrm), **queens** (Q) and **non-reproductive females** (nrf). Squares represent mean of %-areas with error bars (with 95% lower and upper confidence interval, CI; $\alpha = 0.05$). Blue arrows show missing peaks of K, nrm, Q and/or nrf. Results are of HS-SPME-GC-MS from three K, five nrm, three Q and six nrf. REPSTAT = reproductive status.

Tab. IV.3.5: Differences of **individuals** of three different families in four urine volatile compounds (peak 33, 36, 37 and 40) **between reproductive status of the females** (Q and nrf). PA = peak area, IS = internal standard; SD = standard deviation, TIC = total ion chromatogram; Mann-Withney *U*-test, significance level $p < 0.05$.

peak	compound	mean of normalized GC-MS TIC (PAs divided by IS (PA/IS))		Mann-Whitney *U*-test	
		Q ($\bar{x} \pm$ SD)	nrf ($\bar{x} \pm$ SD)	P (n = 8)	U
33	similar to quinoline ?	79.7 ±17.7	37.5 ± 19.4	**0.036**	0.0
36	α-vatirenene?	54.5 ± 24.2	24.6 ± 8.0	0.071	1.0
37	?	56.8 ± 30.2	42.2 ± 46.5	0.571	5.0
40	β-vatirenene or E-farnesene epoxide	92.8 ± 60.9	42.0 ± 26.2	0.250	3.0

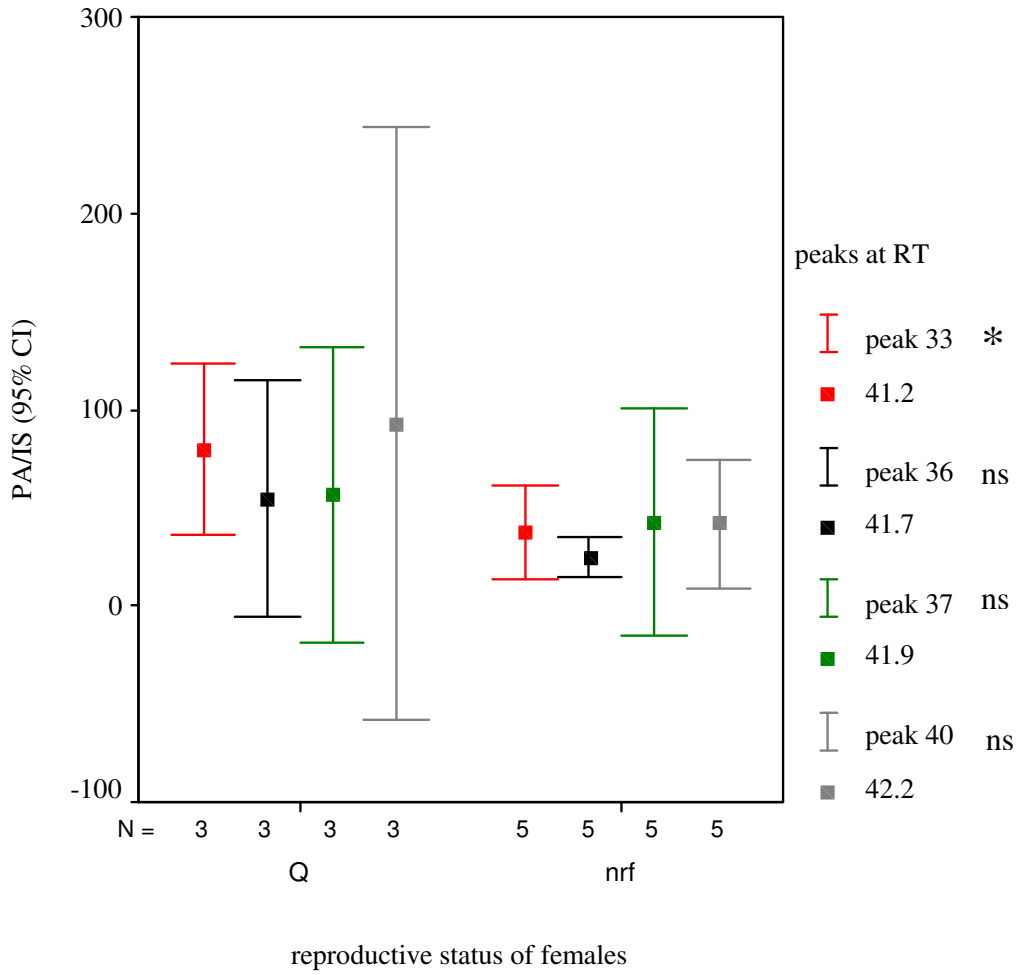


Fig. IV.3.8: Differences of HS-SPME-GC-MS data in **the total concentrations** of four volatile urinary compounds at peak 33, 36, 37 and 40 **between queens** (Q) and **non-reproductive females** (nrf). Squares represent mean of %-areas with error bars (with 95% lower and upper confidence interval, CI; $\alpha = 0.05$). RT = retention time (min), PA = peak area, IS = internal standard, N = number of individuals, * = significant difference ($p < 0.05$), ns = not significant (Mann-Withney *U*-test).

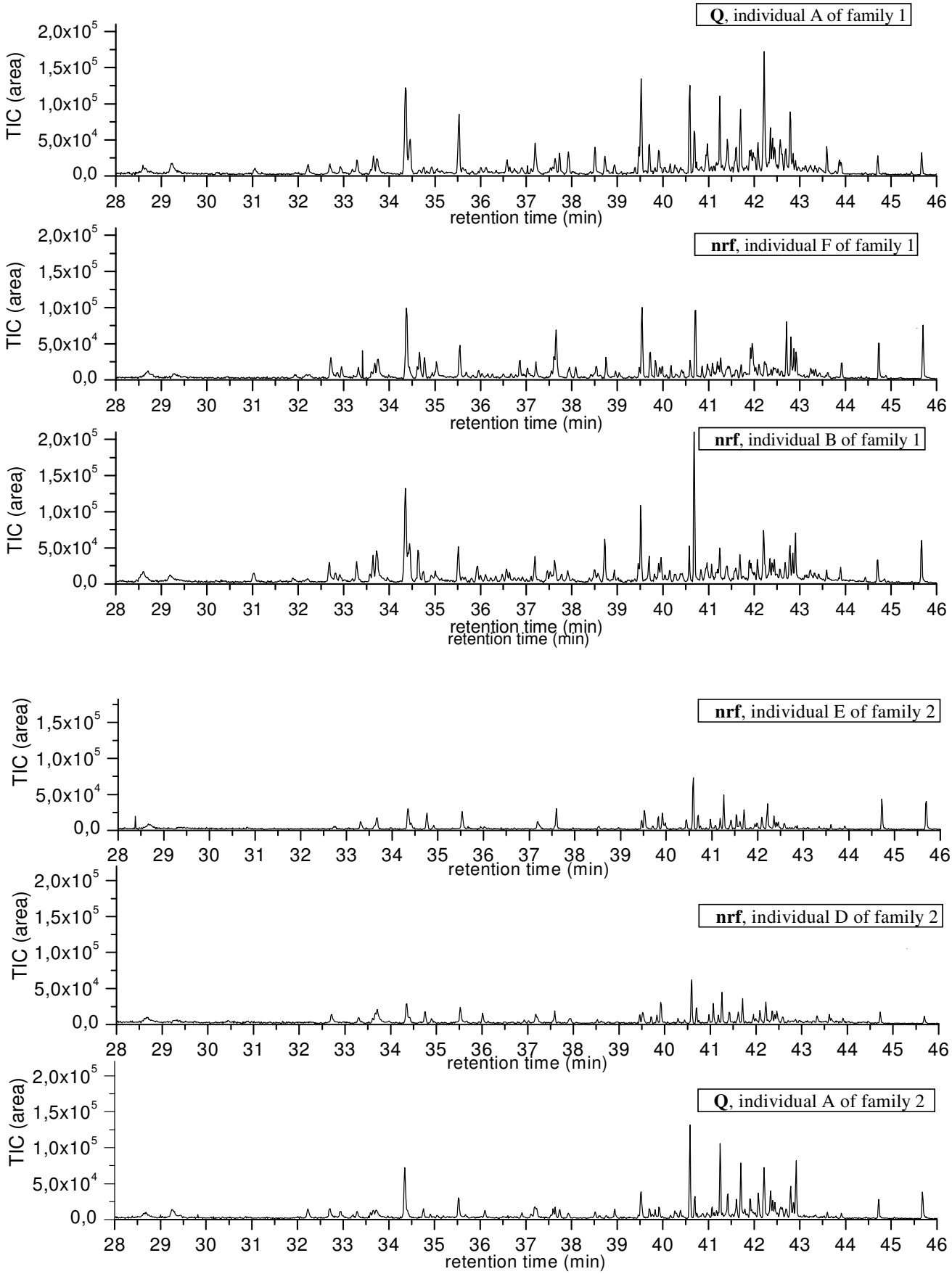


Fig. IV.3.9a-b: TICs (total ion chromatograms) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of urine samples from female individuals of **family one and family two** of Ansell's *Fukomys* mole-rats illustrating **similarity** between **Q** and **differences** compared to their **daughters** urinary peak profiles (Q = queen, nrf = daughter).

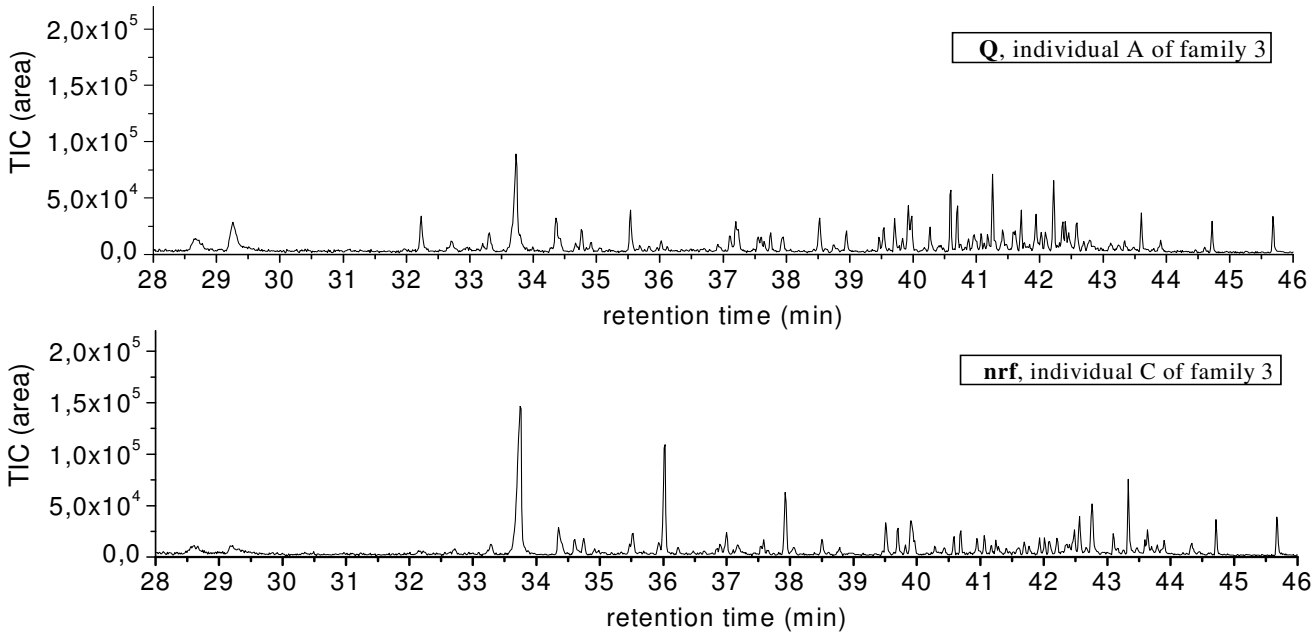


Fig. IV.3.9c: TICs (total ion chromatograms) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of urine samples from female individuals of **family three** of Ansell's *Fukomys* mole-rats illustrating **similarity** between **Q** and **differences** compared to their **daughters** urinary peak profiles (Q = queen, nrf = daughter).

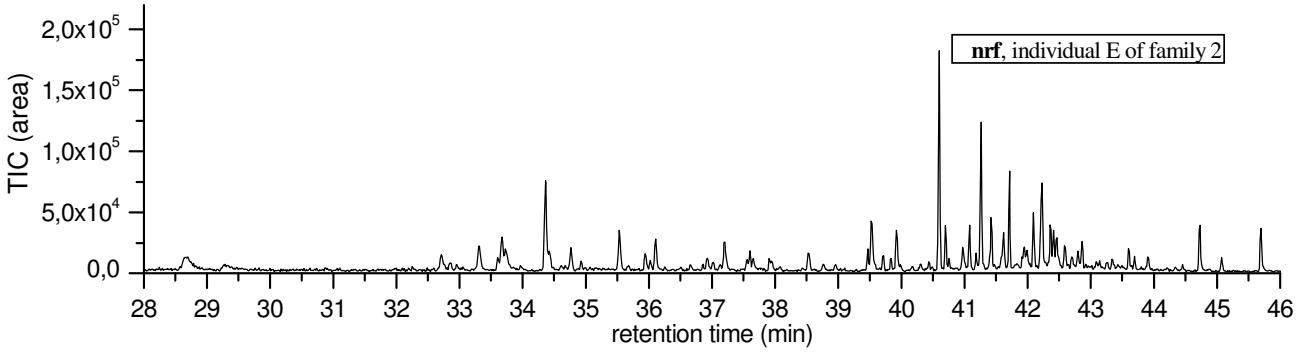


Fig. IV.3.9d: TIC (total ion chromatogram) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from the non-reproductive active **female E** of **family two** of an Ansell's *Fukomys* mole-rat illustrating **similarity** to the urinary peak profiles of **queens** (nrf = daughter).

IV.3.3.6 Differences between queens and all family members

The data from all the families demonstrate that total concentrations of three out of four queen-specific components (cf., chapter IV.3.1.3.5), peak 33, 36 and 40, were significantly higher in queens than in any other family members (K, nrf and nrm) (Mann-Whitney *U*-test; **peak 33**: n = 15, p = 0.004, U = 0.0, mean Q = 79.7 (PA/IS), mean males and nrf = 28.8 (PA/IS); **peak 36**: n = 15, p = 0.011, U = 1.0, mean Q = 54.6 (PA/IS), mean males and nrf = 19.1 (PA/IS) and **peak 40**: n = 15, p = 0.031, U = 3.0, mean Q = 92.8 (PA/IS), mean males and nrf = 30.3 (PA/IS)). Note that the king from family one did not have the component at peak 36 at all (tab. IV.3.6, below; fig 3.10). The compound at RT = 41.9 min (peak 37) was higher in queens than in other family members, but the difference being, however, not significant. One nrf (individual F of family one) had an extraordinarily high level of that peak of 110 (PA/IS) (Mann-Whitney *U*-test; peak 37: n = 15, p = 0.004, U = 6.0, mean Q = 56.9 (PA/IS), mean males and nrf = 29.5 (PA/IS).

Tab. IV.3.6: Differences between **individuals** of three different families in four urine volatile compounds (peak 33, 36, 37 and 40) **between queens and other family members**. PA = peak area, IS = internal standard; SD = standard deviation, TIC = total ion chromatogram; Mann-Withney *U*-test, significance level $p < 0.05$.

peak	compound	mean of normalized GC-MS TIC (PAs divided by IS (PA/IS))		Mann-Whitney *U*-test	
		Q ($\bar{x} \pm$ SD)	all males and nrf ($\bar{x} \pm$ SD)	P (n = 15)	U
33	similar to quinoline ?	79.7 ± 17.7	28.9 ± 17.9	**0.004**	0.0
36	α-vatirenene?	54.6 ± 24.2	19.1 ± 9.7	0.011 (n = 14)	1.0
37	?	56.9 ± 30.2	29.5 ± 31.0	0.101	6.0
40	β-vatirenene or E-farnesene epoxide	92.8 ± 60.9	30.0 ± 20.2	0.031	3.0

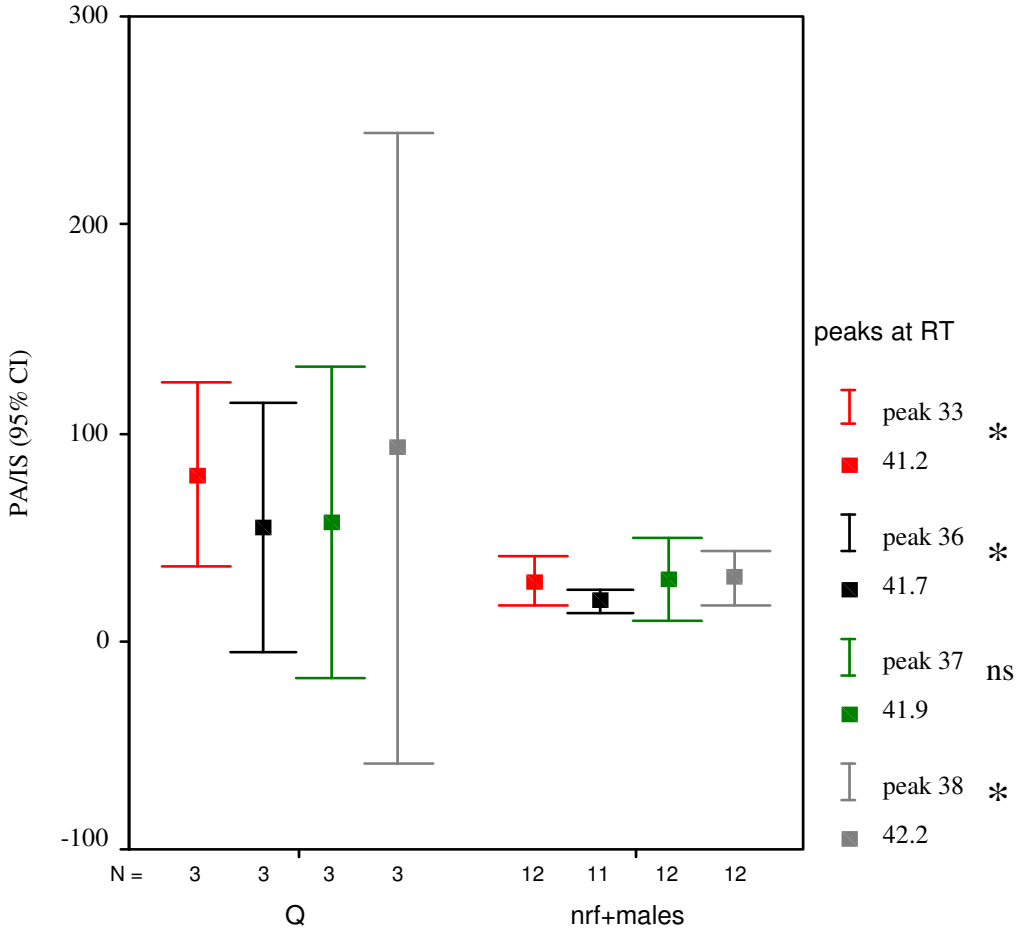


Fig. IV.3.10: Differences of HS-SPME-GC-MS data in **the total concentrations** of four volatile urinary compounds at peak 33, 36, 37 and 40 **between queens** (Q) and **all family members** (K, nrm and nrf). Squares represent mean of %-areas with error bars (with 95% lower and upper confidence interval, CI; $\alpha = 0.05$). RT = retention time (min), PA = peak area, IS = internal standard, N = number of individuals, * = significant difference ($p < 0.05$), ns = not significant (Mann-Withney *U*-test).

IV.3.3.7 Differences between kings and non-reproductive males

No general individual variations were found between males in the TIC, except of occasionally occurring single peaks. No pattern, differing kings from sons could be detected (fig. IV.3.7). Peak 8 at RT = 33.7 min and peak 9 at RT = 34.3 min seem to be higher in nrm than in K, but large variations in the height of these peaks between males of the same reproductive state causes the large error bars, e.g. the values of peak 8 in the individuals of family three: It ranges from 1.6 %-area in the king to 19.3 %-area in nrm C.

IV.3.3.8 Differences between females and males

Males clearly can be distinguished from females by one component; peak 24 at RT = 39.7 min is significantly higher in males than in females irrespective of their reproductive status (Mann-Whitney *U*-test, n = 16, p = 0.005, U = 6.0, mean males = 121.7 (PA/IS), mean females = 41.8 (PA/IS)). Furthermore peak 23 at RT = 39.5 min is tendentially higher in males than in females (Mann-Whitney *U*-test, n = 16, p = 0.606, U = 26.0, mean males = 77.4 (PA/IS), mean females = 59.5 (PA/IS), tab. IV.3.7 below; fig. IV.3.11). Both components were not affected by carrot or potato diet. By mass spectra and co-injection, the component 24 could clearly be identified as ß-caryophyllene. Peak 23 was identified by mass spectra as 2-methyl-4-vinylphenol (see below; tab. IV.3.7).

Furthermore, four components could be categorized to be female typical peaks. Females, irrespective of their reproductive status showed these peaks which were not detectable in males (K or nrm); peak 5 at RT = 32.9 min was found in low amount in Q and nrf (0.5 – 1.3 %-area). It was not found in the queen of family three and it could be detected in just two of six nrf. Peak 6 at RT = 33.2 min was also found in low amount in Q and nrf (0.1 – 0.4 %-area) (fig. IV.3.7). This peak was present in one nrm with 0.1 %-area. At RT = 42.7 min, a component at peak 44 was present in all queens and three nrf (0.5 - 2.9 %-area) in low amounts. Notably, three nrm had a peak at this RT but with completely different mass spectra. Component at RT = 42.8 min, peak 45, was present in all queens and three nrf (0.9 – 4.1 %-area). It was present in one nrm with a %-area of 1.4 (fig. IV.3.7).

Tab. IV.3.7: Differences between **individuals** of three different families in two urine volatile compounds (peak 23 and 24) **between sexes** (all males and females). PA = peak area, IS = internal standard, SD = standard deviation, TIC = total ion chromatogram; Mann-Withney *U*-test, significance level $p < 0.05$.

peak	compound	mean of normalized GC-MS TIC (PAs divided by IS (PA/IS))		Mann-Whitney *U*-test	
		males ($\bar{x} \pm$ SD)	females ($\bar{x} \pm$ SD)	P (n = 16)	U
23	2-methoxy-4-vinylphenol	77.4 ± 52.5	59.5 ± 43.9	0.606	26.0
24	ß-caryophyllene	121.7 ± 70.5	41.8 ± 23.7	**0.005**	6.0

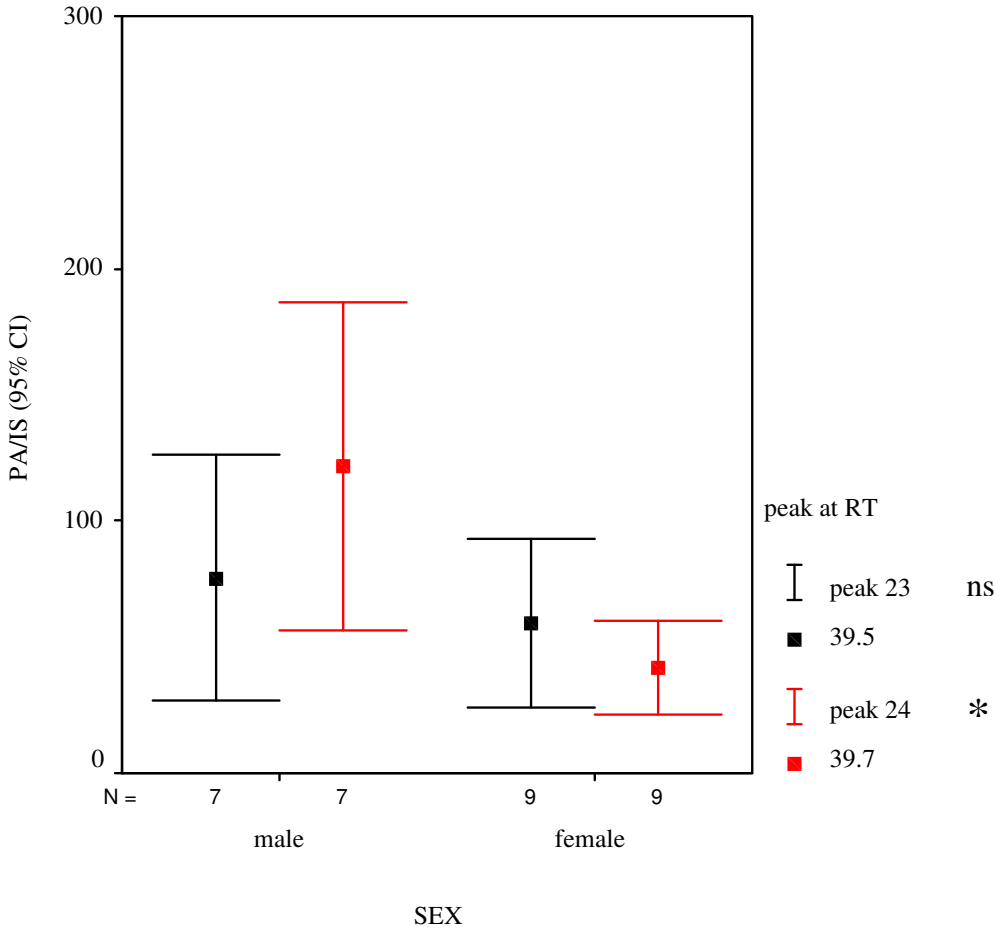


Fig. IV.3.11: Differences of HS-SPME-GC-MS data in the **total concentrations** of two volatile urinary compounds at peak 23 and 24 **between males** and **females** (irrespective of reproductive status). Squares represent mean of %-areas with error bars (with 95% lower and upper confidence interval, CI; $\alpha = 0.05$). RT = retention time (min), PA = peak area, IS = internal standard, CI = confidence interval, N = number of individuals, * = significant difference ($p < 0.05$), ns = not significant (Mann-Withney *U*-test).

IV.3.3.9 MS-Spectra

Discovered compounds in the TIC of GC were only accepted, if the identification with mass spectra and the data of the NIST library (2002) showed a match above 900.

The compound ß-caryophyllene (peak 20 in the TIC of families according to peak 24 in the TIC of individuals) could be confirmed additionally to urinary volatile samples by co-injection of ß-caryophyllene in ddH_2O as external standard. Like in urinary volatile samples, the retention time was the same, at RT = 39.7 min. The mass spectrum demonstrated a very high match (928) to the reference substance of the NIST library (2002) (appendix fig. VII.C10). The probability was even higher in urinary volatiles of animals, e.g. purity of the sample from individual nrm C of family two was 96 % and the match was 943 (NIST 2002; fig. IV.3.12 a,b).

Further four components could clearly be identified by references of the NIST library. Moreover, five components were tentatively specified with high probability by the NIST library: Peak 3 at RT = 32.3 min could be identified best in the queen of family three as 4-nonanone, with a match of 902 and a probability of 58.1 % (NIST 2002; fig. IV.3.13 a,b). Furthermore, peak 4 could be specified tentatively as (E)-2-octenal, with high match of 874 in individual nrm D of family one, but further analytical work is necessary to confirm the finding. The components at RT = 33.6 and 33.7 min were not well separated from the urinary mixture of families and revealed an overlap, both comprising peak 6. The subsequent GC-separation of individual urinary volatiles delivered better purity and identification than the GC-separation of urine from families: The original peak 6 of the urine from families could be separated into two peaks and finally was classified as peak 7 and 8 (of the urine from individuals). Peak 7 at RT = 33.6 min could be identified as 1,3,8-p-menthatriene (individual nrf F, family two; purity of 68 %, match of 866 and a probability of 19.6 %) and peak 8 at RT = 33.7 min was analysed best in the individuals of family three, since they have very high abundance of that component. It could be identified by mass spectra as 4-methylphenol (individual nrf C, family three, purity of 89 %, match of 931 and a probability of 60 %) (fig. IV.3.14 a,b).

Peak 9 at RT = 34.3/4 min (corresponding to peak 7 of family data) showed the mass spectrum of acetophenone with a purity of 92 % and a match to the reference of NIST (2002) of 944 (nrm D, family one, fig. IV.3.15 a,b).

Peak 23 at RT = 39.5 min contributed to high %-areas (13.0) in males of family one and could be classified best in urinary volatiles of individual nrm D as 2-methoxy-4-vinylphenol, with purity of 94 %, a match of 899 and probability of 58.9 %. Even if the match is below 900, it is highly likely that this compound is 2-methoxy-4-vinylphenol (fig. IV.3.16 a,b).

Peak 47 at RT = 43.3/4 min (corresponding to 37 of family data) could be verified as 1-hexadecanol best by urinary volatiles from individual nrf C of family three with purity of 88%, a match of 911 and probability of 12.6 % (fig. IV.3.17 a,b). This peak occurred only in non-reproductive females. Peak 50 at RT = 44.7 min (corresponding to peak 40 of family data) was present in every individual and most probably can be specified as hexadecanoicacidbutylester, but since its match is below 900 (individual nrf F, family two, match 818, probability 80.4 % and a purity of 87 %), without further confirmation this remains tentative (fig. IV.3.18 a,b). The same situation was present in peak 51 (corresponding to 41 of family data). It was present in every individual and could be specified with high probability, but without further confirmation: Again, individual nrf F of family two showed the best result, identifying the compound as octadecanoicacidbutylester. The match is 826 with purity of 88 % and a probability of 70.4 % (fig. IV.3.19 a,b).

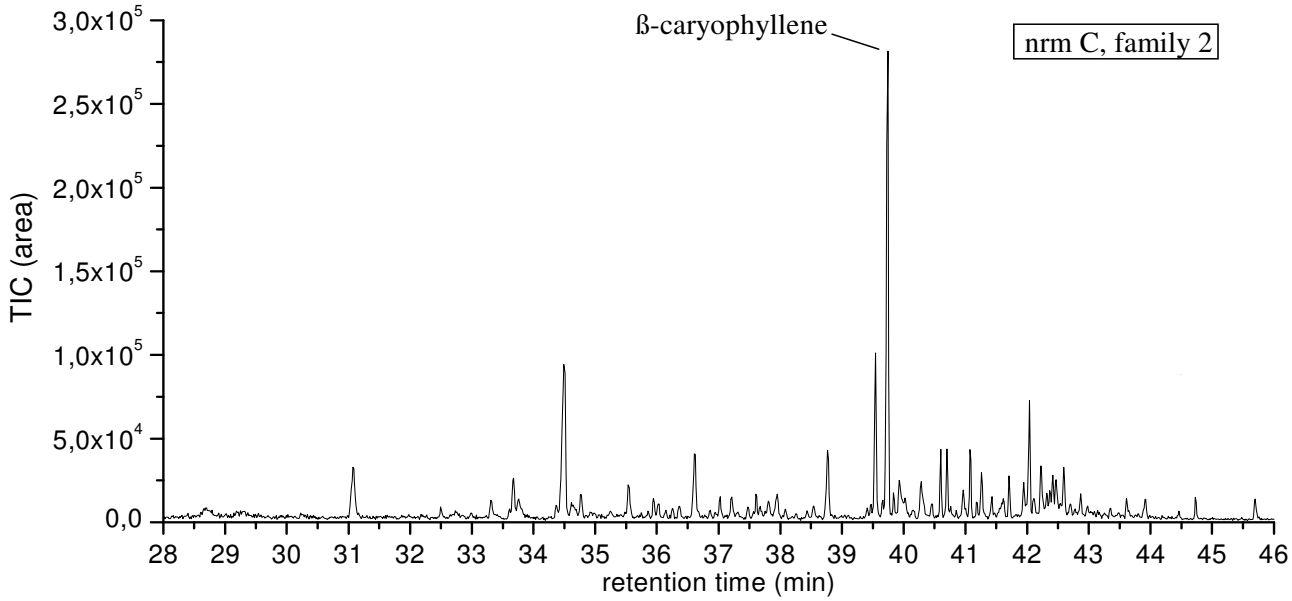


Fig. IV.3.12a: **TIC** (total ion chromatogram) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from individual nrm C of family two of an Ansell's *Fukomys* mole-rat illustrating high abundance of **ß-caryophyllene** (peak 24 at RT = 39.7 min).

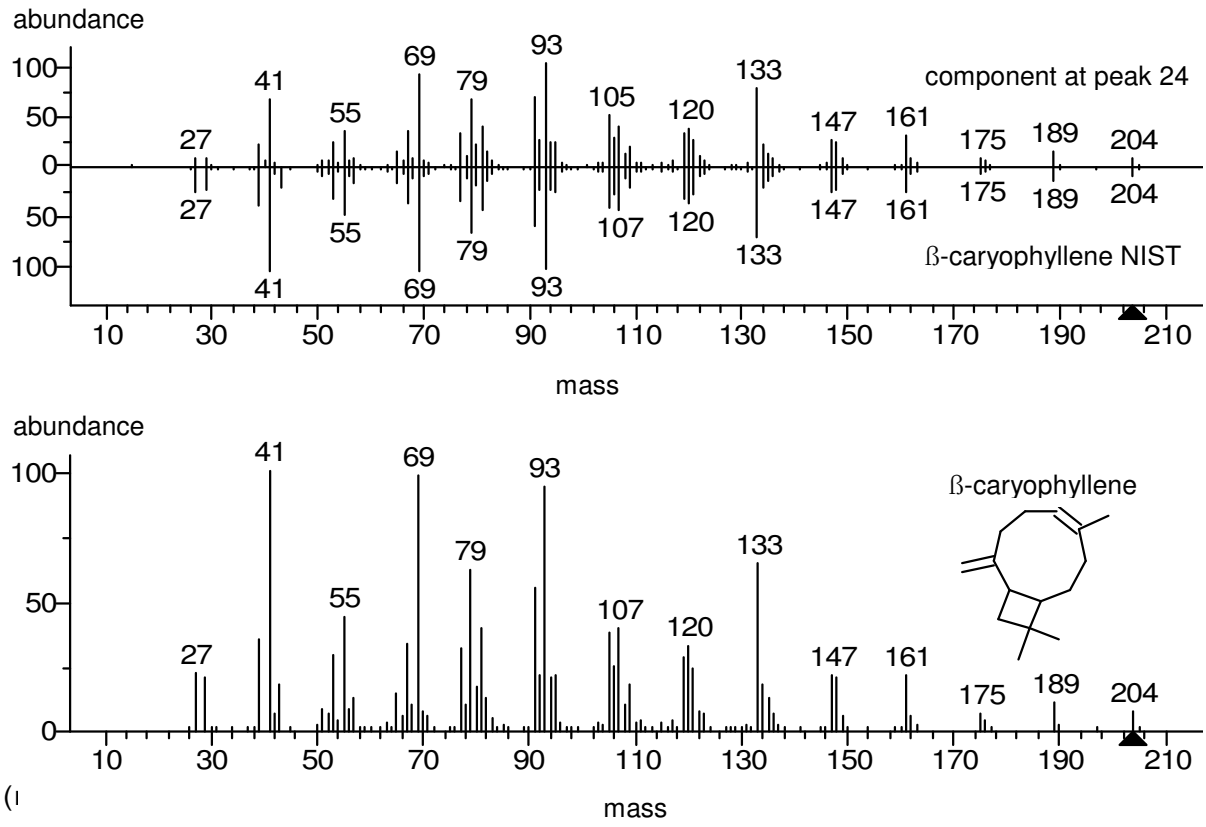


Fig. IV.3.12b: Mass spectral identification of ß-caryophyllene (peak 24 at RT = 32.2 min) confirmed by NIST library of individual nrm C of family two. Above, head-to-tail presentation of component at peak 24 to the reference substance ß-caryophyllene of NIST (2002).

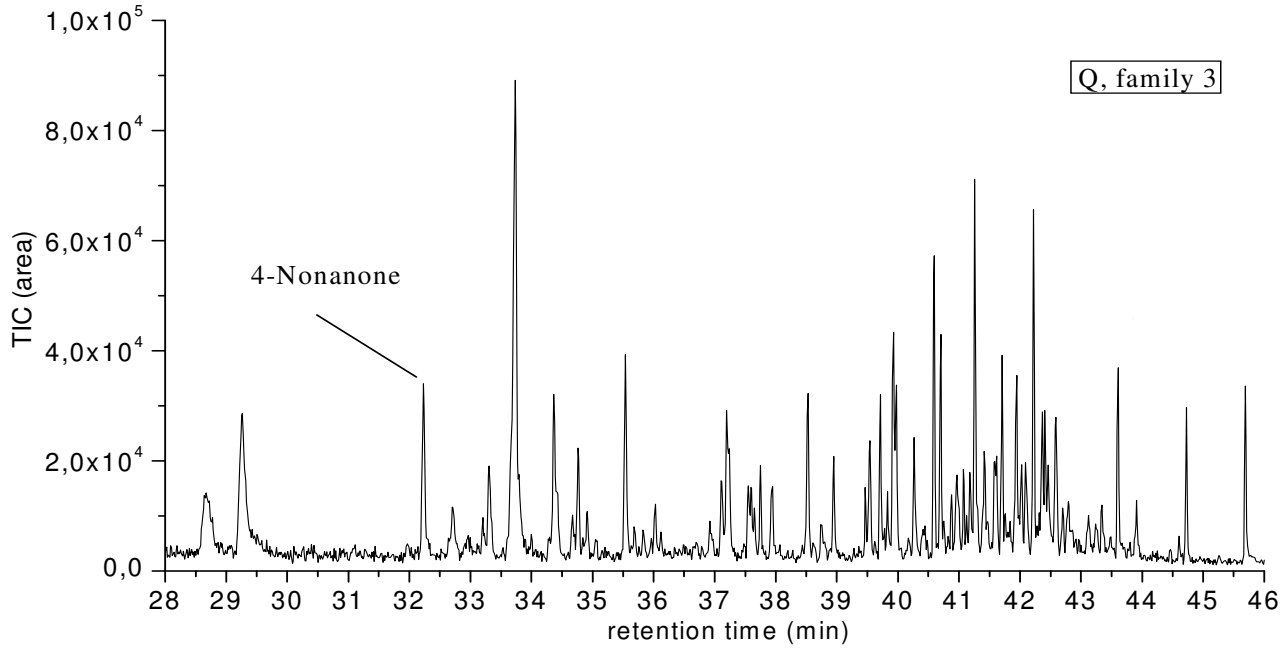


Fig. IV.3.13a: **TIC** (total ion chromatogram) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from the queen (Q) of family three of an Ansell's *Fukomys* mole-rat illustrating **queen-specific compound, 4-nonanone**; peak 3 at RT = 32.3 min.

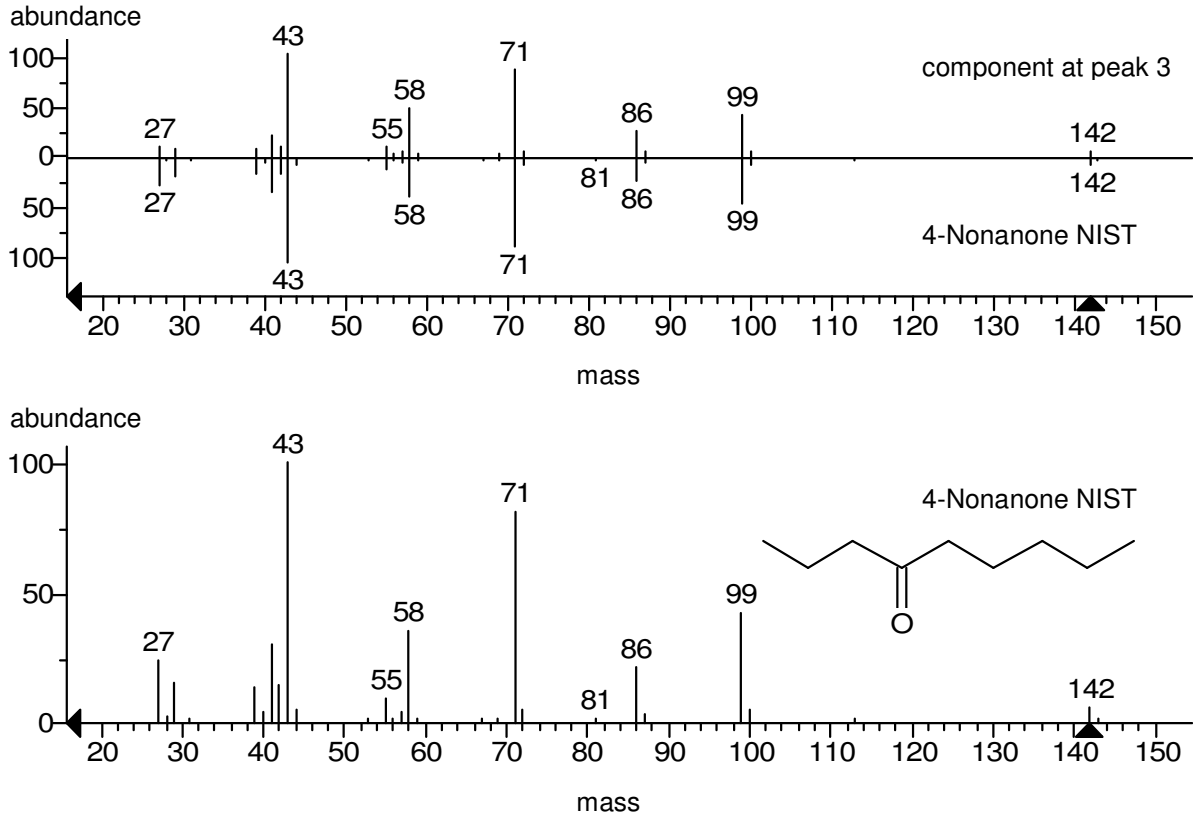


Fig. IV.3.13b: **Mass spectral identification of 4-nonanone** (queen-specific peak 3 at RT = 32.2 min) from the queen of family three, confirmed by NIST library. Above, head-to-tail presentation of component at peak 3 to reference substance 4-nonanone of NIST (2002).

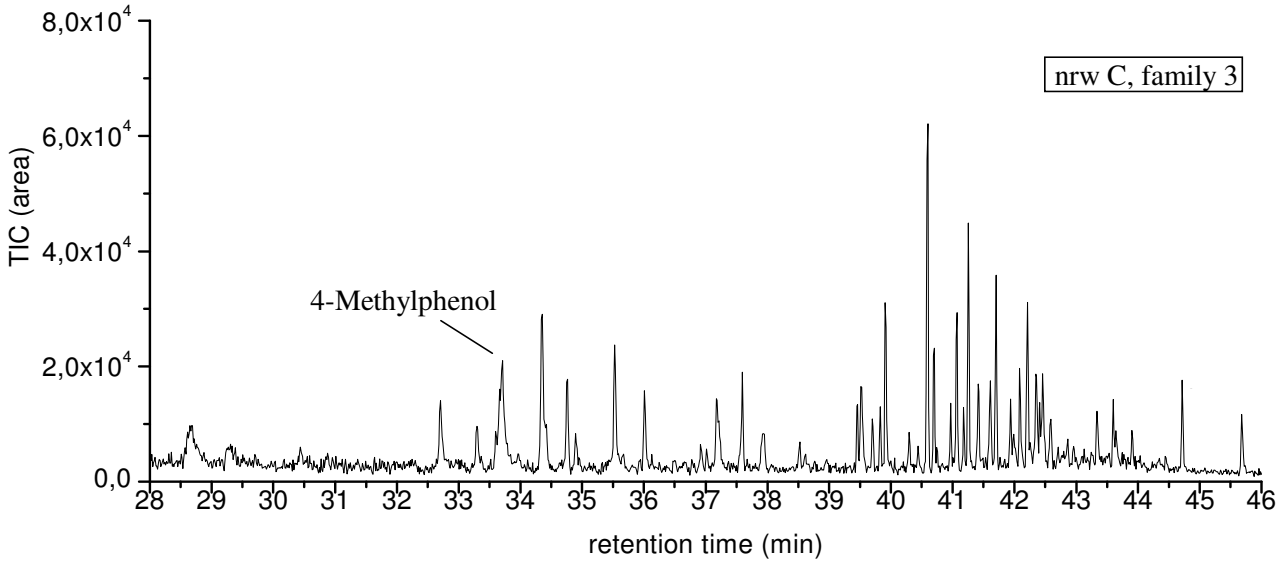


Fig. IV.3.14a: TIC (total ion chromatogram) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from individual nrf C of family three of an Ansell's *Fukomys* mole-rat illustrating **high abundance of 4-methylphenol**, peak 8 at RT = 33.7 min.

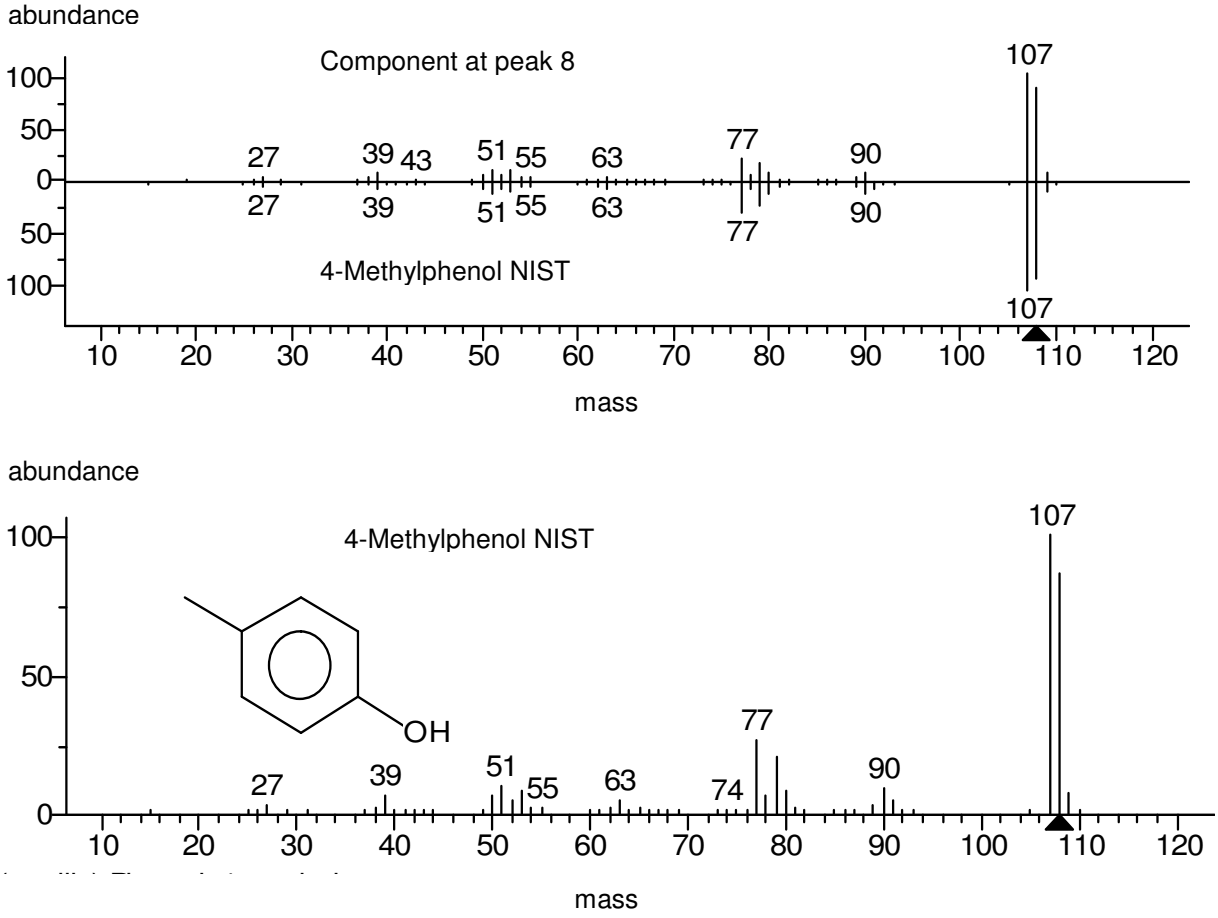


Fig. IV.3.14b: Mass spectral identification of 4-methylphenol (peak 8 at RT = 33.7 min) confirmed using NIST library of individual nrf C of family three. Above, head-to-tail presentation of component at peak 8 to reference substance 4-methylphenole of NIST (2002).

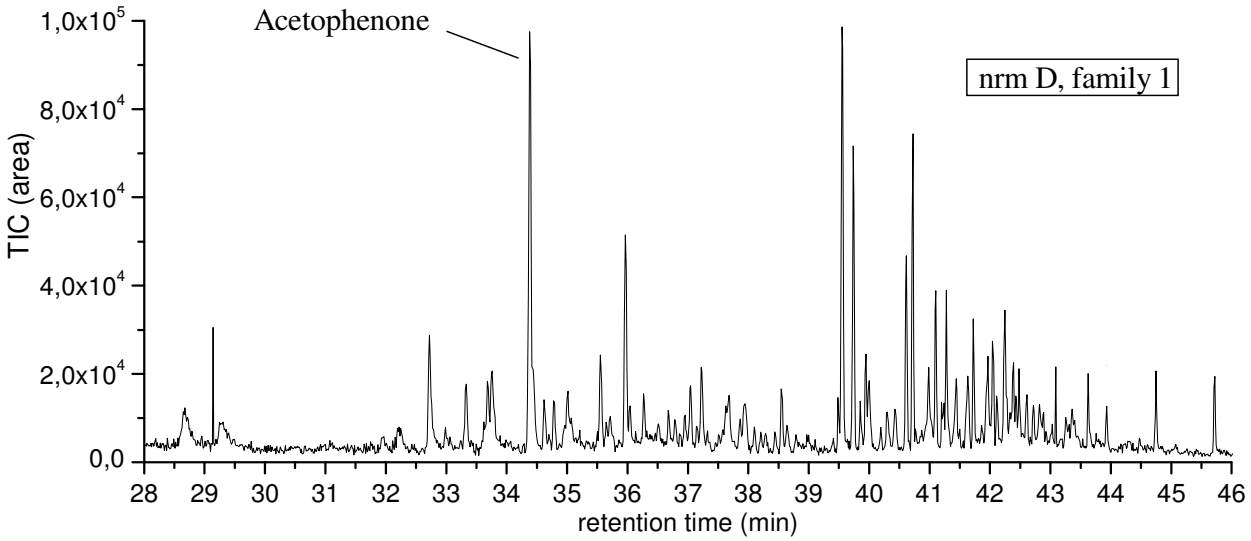


Fig. IV.3.15a: TIC (total ion chromatogram) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from individual nrm D of family one of an Ansell's *Fukomys* mole-rat **illustrating high abundance of acetophenone**, peak 9 at RT = 34.3/4 min.

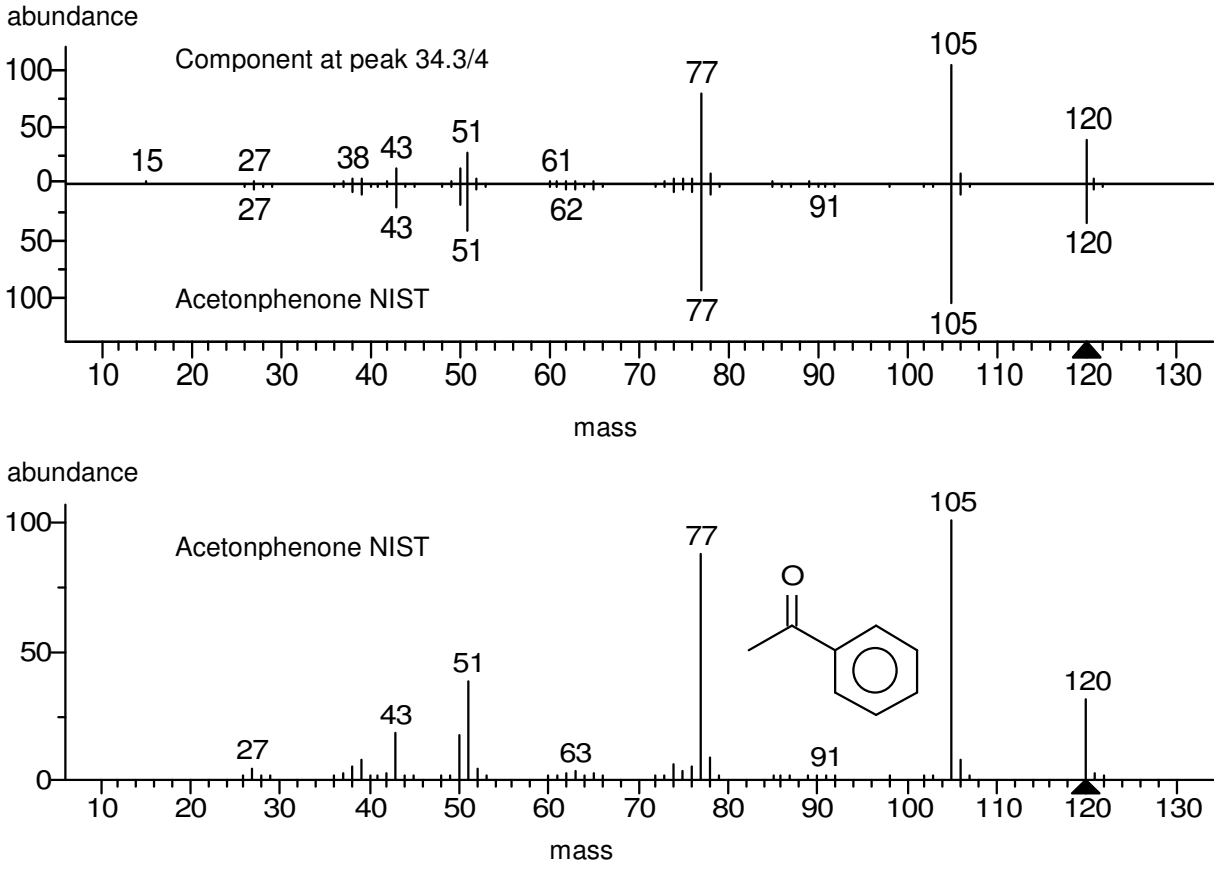


Fig. IV.3.15b: Mass spectral identification of acetophenone (peak 9 at RT = 34.3/4 min) confirmed using NIST library of individual nrm D of family one. Above, head-to-tail presentation of component at peak 9 to reference substance acetophenone of NIST (2002).

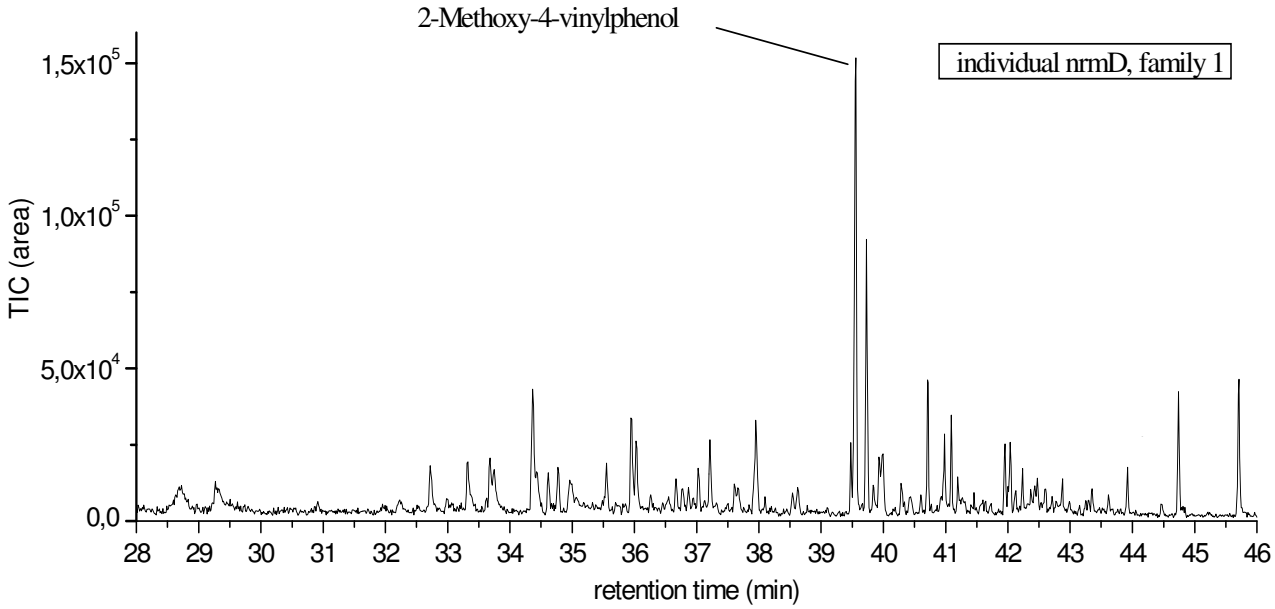


Fig. IV.3.16a: TIC (total ion chromatogram) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from individual nrm D of family one an Ansell's *Fukomys* mole-rat **illustrating high abundance of 2-methoxy-4-vinylphenol**, peak 9 at RT = 34.3/4 min.

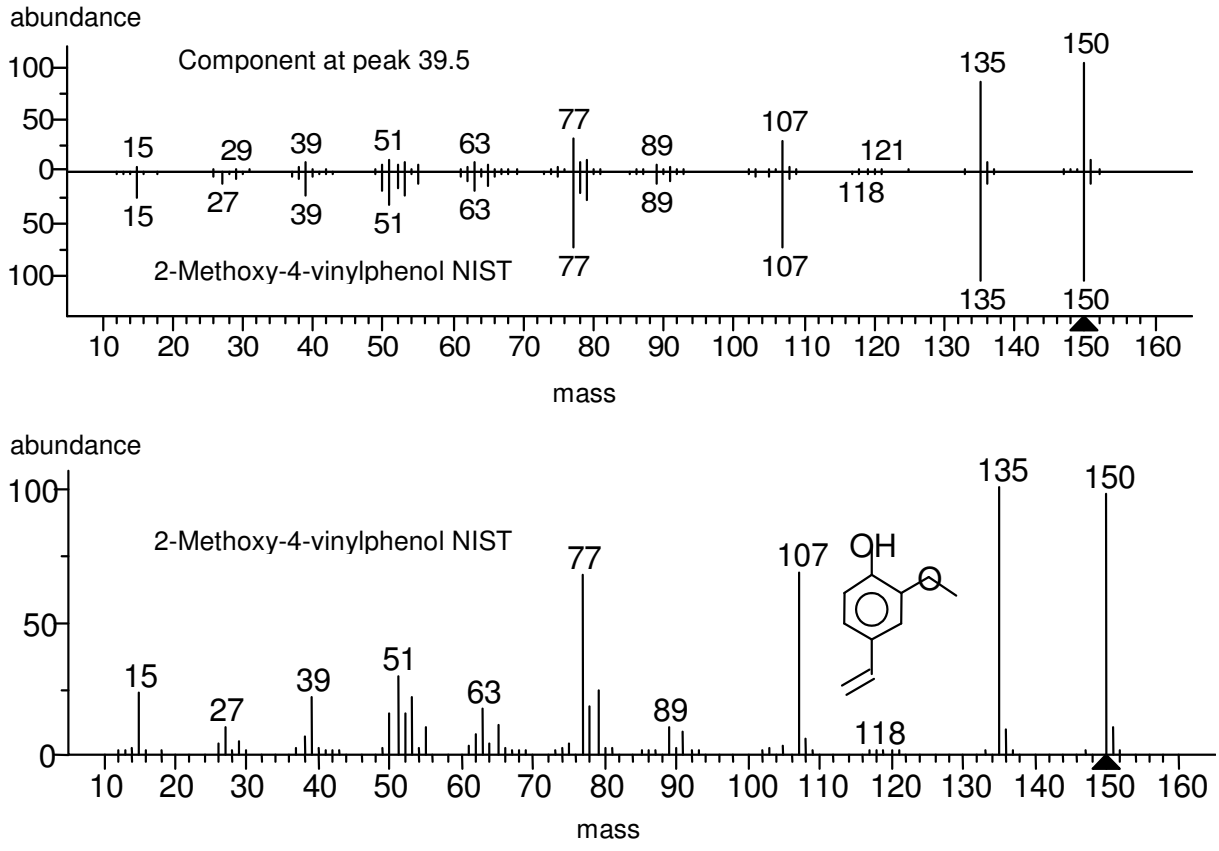


Fig. IV.3.16b: **Mass spectral identification of 2-methoxy-4-vinylphenol** (peak 9 at RT = 34.3/4 min) confirmed using NIST library of individual nrm D of family one. Above, head-to-tail presentation of component at peak 9 to reference substance 4-methoxyvinylphenol of NIST (2002).

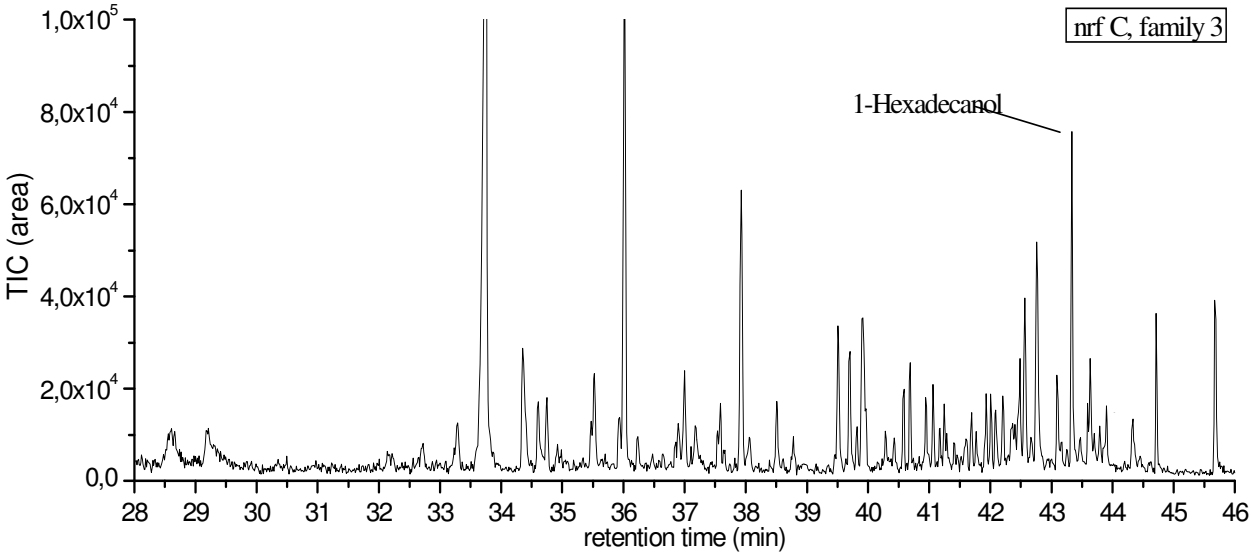


Fig. IV.3.17a: TIC (total ion chromatogram) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from individual nrf C, family three of an Ansell's *Fukomys* mole-rat **illustrating high abundance of 1-hexadecanol**, peak 47 at RT = 43.3/4 min.

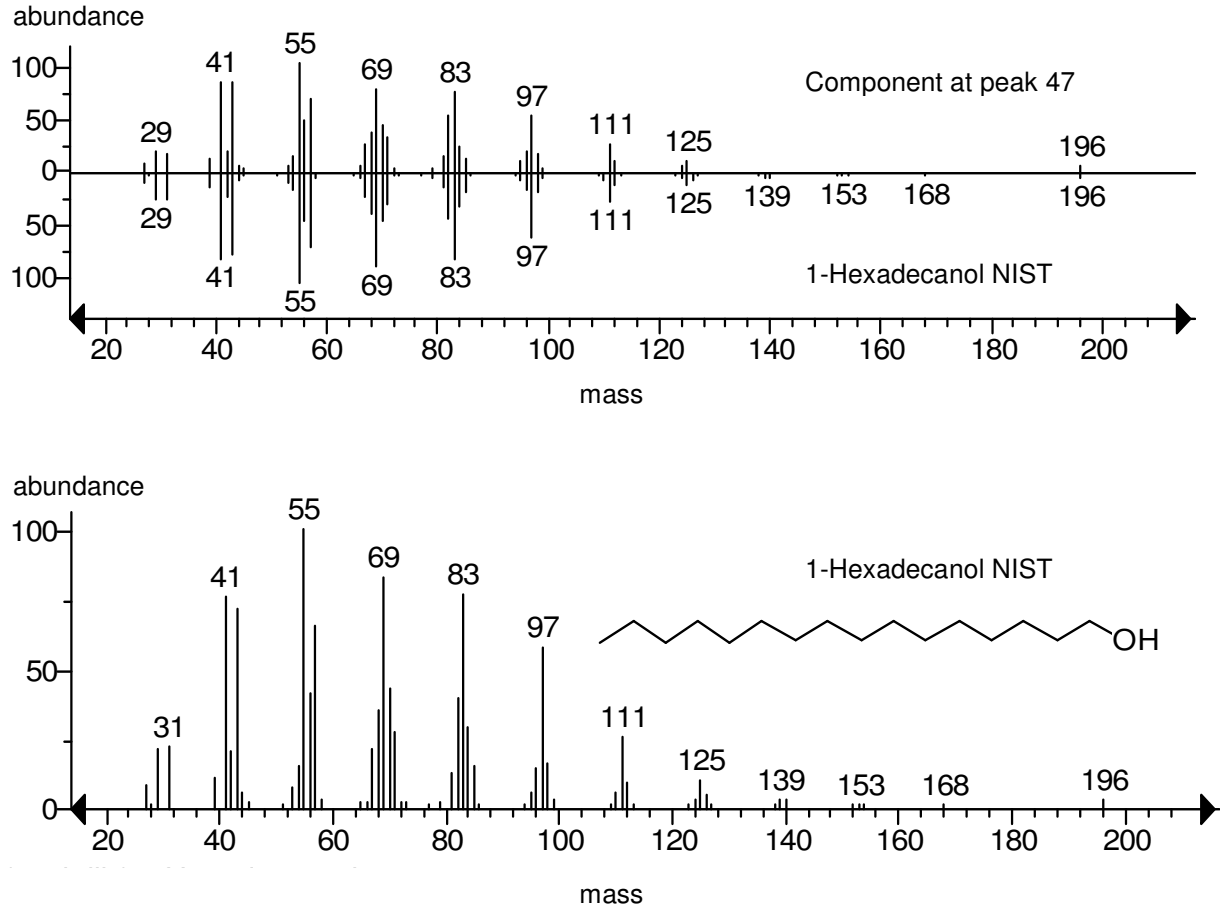


Fig. IV.3.17b: Mass spectral identification of 1-hexadecanol (peak 47at RT = 43.3/4 min) confirmed using NIST library of individual nrf C from family three. Above, head-to-tail presentation of component at peak 47 to reference substance 1-hexadecanol of NIST (2002).

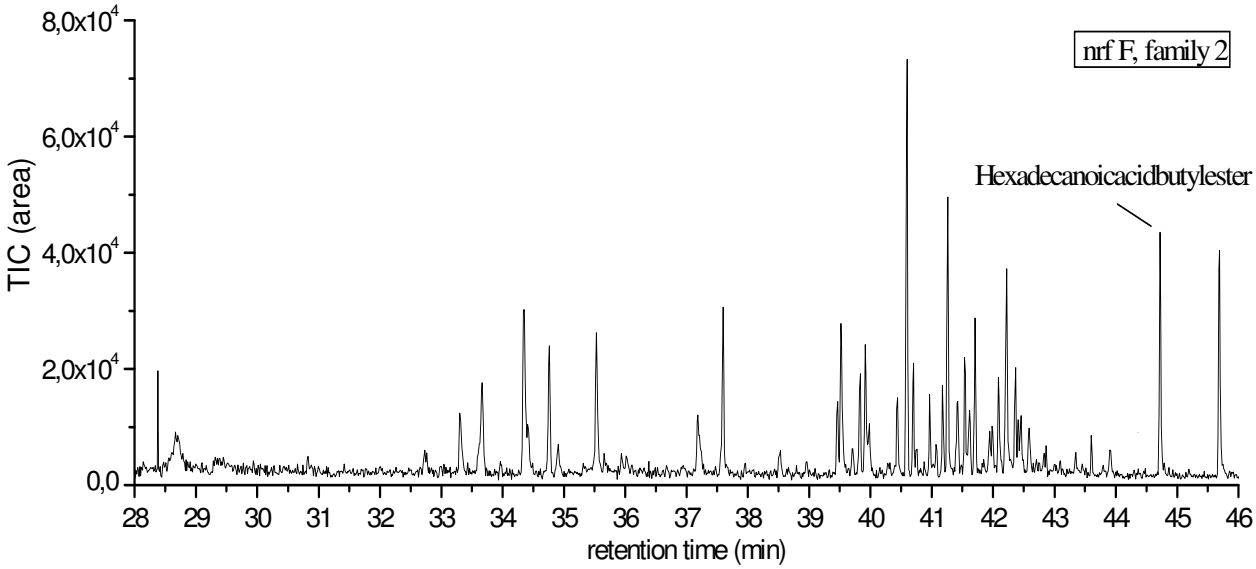


Fig. IV.3.18a: TIC (total ion chromatogram) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from individual of nrf F, family two of an Ansell's *Fukomys* mole-rat illustrating **hexadecanoicacidbutylester**, peak 50 at RT = 44.7 min.

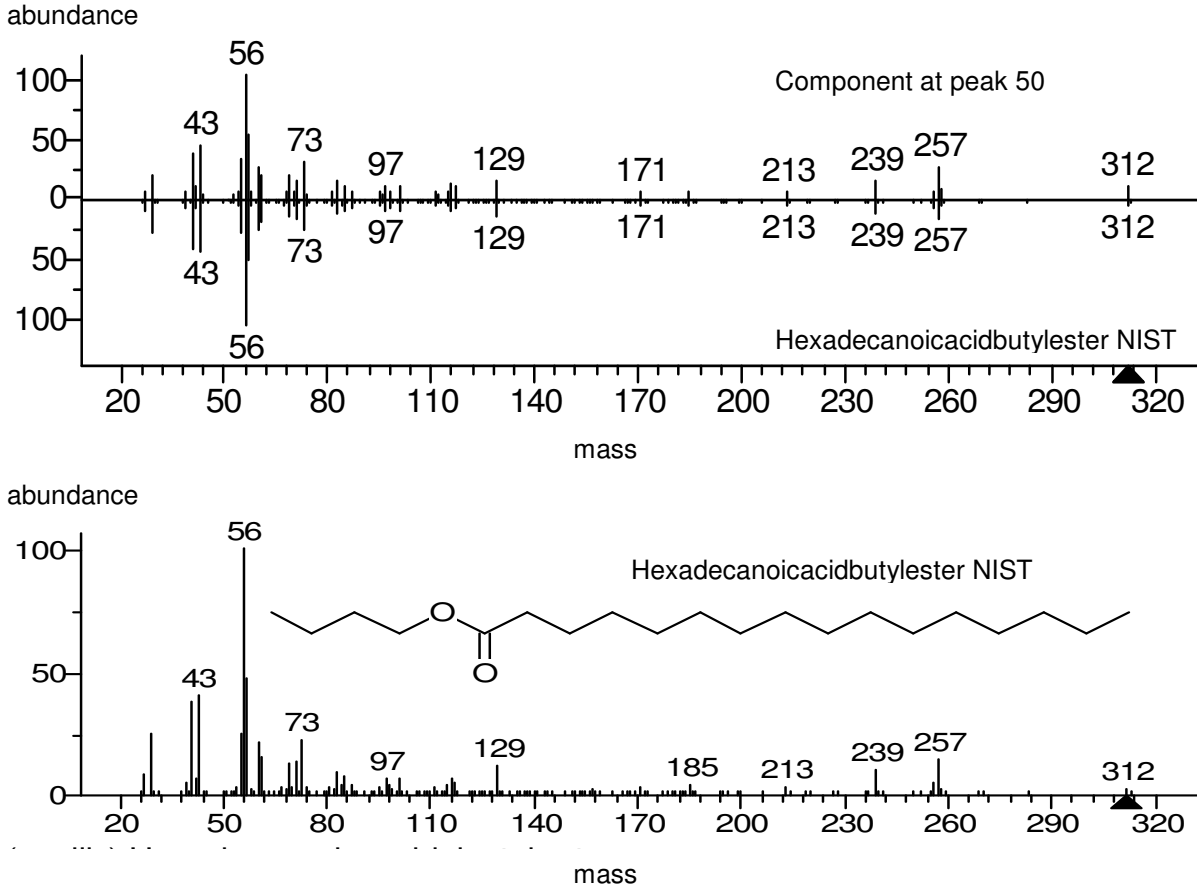


Fig. IV.3.18b: Mass spectral identification of hexadecanoicacidbutylester (peak 50 at RT = 44.7 min) confirmed using NIST library of individual nrf F from family two. Above, head-to-tail presentation of component at peak 50 to reference substance hexadecanoicacidbutylester of NIST (2002).

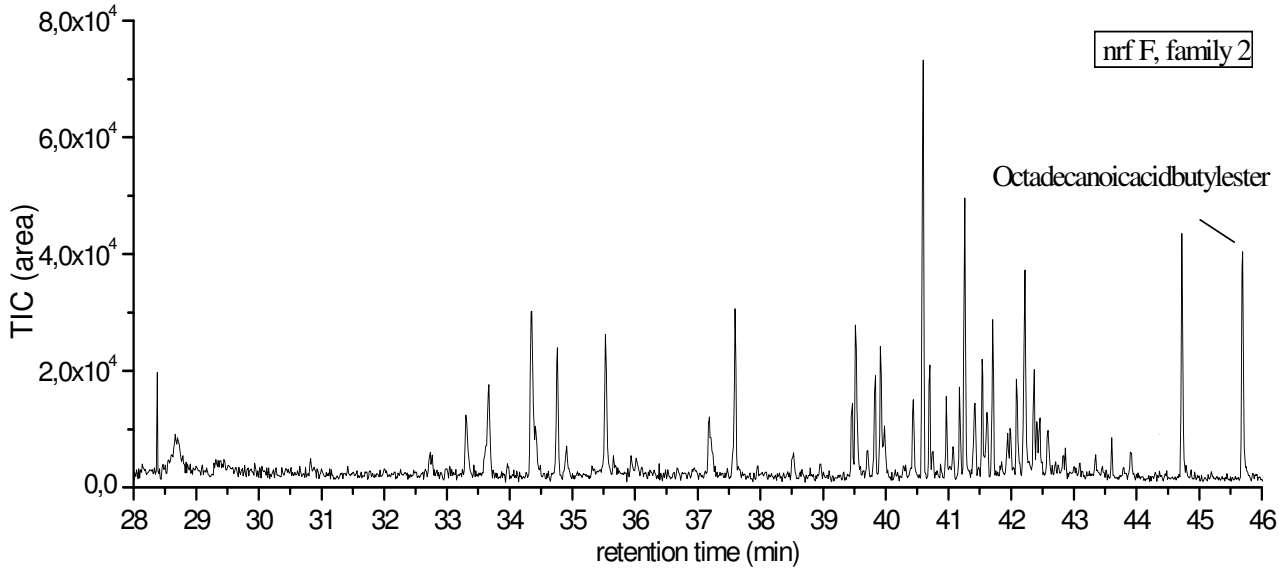


Fig. IV.3.19a: TIC (total ion chromatogram) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from individual nrf F, family two of an Ansell's *Fukomys* mole-rat illustrating **octadecanoicacidbutylester,** peak 51 at RT = 45.7 min.

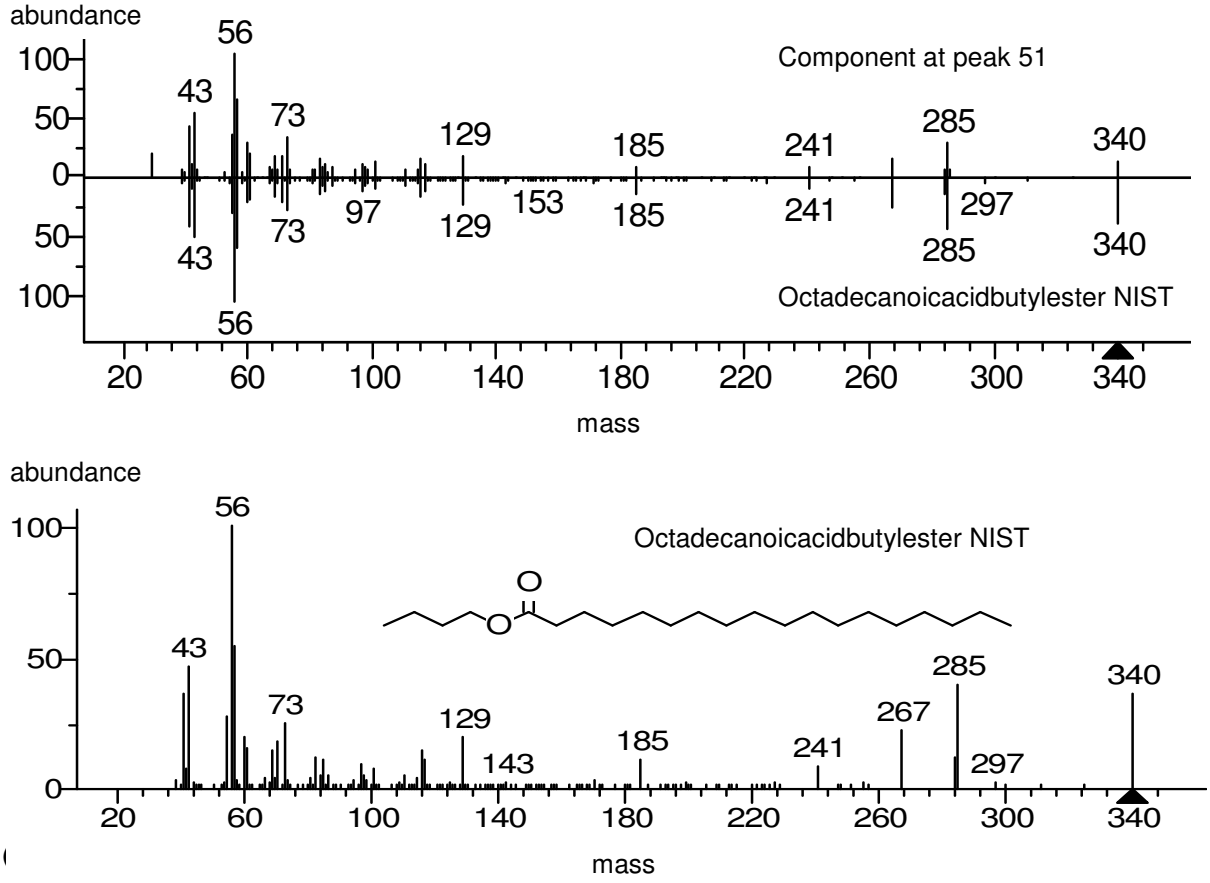


Fig. IV.3.19b: Mass spectral identification of octadecanoicacidbutylester (peak 51 at RT = 45.7 min) confirmed using NIST library of individual nrf F from family two. Above, head-to-tail presentation of component at peak 51 to reference substance octadecanoicacidbutylester of NIST (2002).

IV.3.4 DISCUSSION

Method

This work demonstrates an innovative approach by which compounds emitted in the head-space of urine can be analysed with the technique of SPME-GC-MS. Here, a reliable and reproducible method was designed, where the chromatogram and mass spectra were sharpest when using a three-component 50/30 µl DVB/CAR™/PDMS™Stable Flex™-fibre by the method of head-space sampling instead of concentrating by immersion. This fibre generally is used for semi-volatile and volatile analyte group flavours (Weber 2003, URL 1). Willse et al. (2006) proved this fibre to be suitable for SPME-GC-MS analysis of *M. m. domesticus* urine as well. Neither the PDMS-fibre for non-polar high molecular weight compounds nor the CAR/PDMS-fibre which Kayali-Sayadi et al. (2003) proved to be successful in adsorbing urinary MUP-ligands of the *M. m. domesticus* were successful in the current study. The method of immersion was always ineffective (cf., appendix tab. VII.C1). The applied sampling procedure produced nearly identical chromatograms, when measuring same specimens repeatedly (appendix fig. VII.C7 a-c), with nearly identical retention times. Simultaneously, marked differences between the chromatograms of individuals of different categories (e.g. queen or non-reproductive females), or families with and without diet were obvious (fig. IV.3.3 and fig. IV.3.5a-d). Calibration with two internal standards, 1-(S)-α-pinene and vertinpropionate, delivered reproducible peaks with same retention times and a high linearity of regression curves (cf., appendix fig. VII.C2, C3 and VII.C7a-c), allowing to consider peak areas as reliable parameters for quantitative calculation (and thus comparing) of concentrations of urinary volatile components between different test categories, i.e. diet, families, individuals, sex, and reproductive status. The "blind search" for compounds in the urine of *Fukomys* mole-rats as possible odourous substances now can be restricted to 54 detected components of which the identification of ß-caryophyllene could be confirmed by both, retention time and mass spectra and also by co-injection of ß-caryophyllene (appendix fig. VII.C10). Further four components (4-nonanone, 4-methylphenole, acetophenone and 1-hexadecanol) could be verified by mass spectra with a match above 900 to the reference compounds of NIST library (2002). One compound, 2-methoxy-4-vinylphenol, was specified by a match of 899, thus it seems very likely that it corresponds to the reference substance of the NIST library (2002). Two further components, hexadecanoicacidbutylester and octadecanoicacidbutylester, were most probably identified. They were always present, regardless of the individual category, but since their matches were 818 and 826, further analytical work (e.g. comparing to RT and mass spectra of external standards) seems to be necessary for identifying it in the urinary volatiles

of *Fukomys* mole-rats. Further 46 unidentified components still await chemical analytical verification.

The current study provides a reliable and reproducible method as a first guideline for further analytical work, delivering at least eight target compounds for different body excretions, e.g. ano-genital odour or volatile body odour. Furthermore, the identified urinary components in this study can be applied as pure analytical compounds in behavioural assays for testing their olfactory and/or pheromonal relevance to *Fukomys* mole-rats.

Family

The urine of *Fukomys* mole-rats is of high chemical complexity. Altogether 41 peaks were detected in the TIC of HS-SPME-GC-MS in every family (except of three components at RTs of 41.1, 41.2 and 41.4 min in some hybrid family samples; cf., tab. IV.3.3), thus probably characterizing the genus-specific volatile urinary profile. The %-areas of the abovementioned components of individual urinary samples of the hybrid family four were low (partly below integration level, see below), thus reflecting the detection limit in mixed volatile urine samples of family profiles. The relative proportion of small peaks was probably dropped down by more pronounced peaks of certain family members or simply by overlap and/or masking effects, causing lower detection limits in the family samples. The component at RT = 41.2 min, for instance, was found in the *Kafuensis*-queen of the hybrid family four in relative higher concentration (%-area) than the other peaks (see below), finally contributing to the detection of this peak in the urinary family pattern. Due to this fact, the undetected peaks (at RT = 41.1, 41.2 and 41.4 min) seem not to contribute to differentiation of e.g. hybrids (*F. anselli* and *F. kafuensis*) from *F. anselli* by urinary volatiles, additionally because the urinary sample mix of family four comprised merely two hybrids (one daughter and one son), whilst the parents were *F. anselli* (king) and *F. kafuensis* (queen). Rather a high statistically significant similarity of the entire urinary profiles between all the six families (and thus at the same time between both sibling-species) was found (cf., fig. IV.3.4). However, the 41 peaks of urinary profile from *Fukomys* mole-rats differ qualitatively and quantitatively from the profile of other species of different genera, e.g. the house mouse (*M. m. domesticus*), containing compounds such as geraniol or 2-*sec*-butyl-4,5-dihydrothiazole (Zhang et al. 2007, Kayali-Sayadi et al. 2003).

The family three seems to be an exception in that it differed from the other *Fukomys* families in three peaks; an extraordinarily large peak 6 at RT = 33.7 min, identified as 4-methylphenol and one unidentified peak at RT = 44.3 min (tab. IV.3.3). Moreover, peak 3, identified as 4-nonanone, which is supposed here to be a queen identifying peak (discussed below), was not present in the urinary mixture of family three. Since urine of that family was not analysed, it remains questionable (but anyhow very likely) that the urine of that queen

contains also 4-nonanone, due to the supposed masking effect in the urinary samples of families. The detection limit of this compound of the urinary family mixture therefore probably was too low. Beside this three exceptions, the general profile of family urinary volatiles was rather similar as far as the retention time and the concentrations of components are concerned.

This result indicates that urine of families does not contribute to family discriminative odours, probably because of a low odourous relevance of urine in this context for *Fukomys* mole-rats. The behavioural impact of this odour source was tested in the habituation-generalization test as well (chapter IV.2). Those results underline and emphasize very well my chemical analytical findings on behavioural level, since it was found that *Fukomys* mole-rats were not able to discriminate individuals originating from different families, neither in the familiar nor in the strangers condition on the basis of urinary odour (cf., fig. IV.1.6 and fig. IV.1.8; chapter IV.1). According to the present results, continuative behavioural investigations with a mixture of urinary family odours should support hitherto findings.

Interesting results revealing the influence of food upon the urinary odorous family profile were obtained on animals kept on potato or carrot diet. When kept on normal diet, the family two showed a totally different TIC-pattern of urinary volatiles compared to carrot or to potato diet (cf., fig. IV.3.4 and fig. IV.3.5a), implying that the composition of urine is certainly affected through individual- and/or family food preference (and, of course, local food availability) and therefore diet obviously acts on their odours. Thus, in addition, in nature mole-rats most probably differ in their odours according to their occurrence, i.e. depending on locally occurring plants, provided that urine is sensed by mole-rats as olfactory information. The carrot diet altered the urinary profile in that it significantly increased peaks 28-32, whilst further six compounds disappeared (tab. IV.3.3). Unfortunately, none of those "carrot-connected" peaks could chemically be analysed. Contrary to the carrot diet, the potato diet did not influence the urinary profile through increase of several peaks. The potato diet led to loss of seven peaks, of which none was identified by mass spectrometry. One peak at RT = 34.6 min emerged in the tested family two only during the potato diet, its chemical identity remains unknown so far. Thus, it is not yet clear and it remains questionable, to what extent further sources of the *Fukomys* odour might be influenced by family and/or individual food preferences. The current study points out, that further analytical work on other body excretions than urine (whose olfactory meaning might be more powerful in certain contexts for *Fukomys* mole-rats), e.g. body odours or ano-genital odours and their dependence on individual diet has to be done. The question of the general

impact of food preference and its contribution to individual and family odours discrimination thus remains to be solved.

Individuals

No marked individual urinary differences could be proved by the HS-SPME-GC-MS analysis, since the pattern of urinary volatiles in the TIC (total ion chromatogram) within non-reproductive males (sons) and within non-reproductive females (daughters) were highly similar. Even the profiles of kings and sons were similar (cf., fig. IV.3.7b). Previous behavioural findings of habitatuation-generalization tests underlay those chemical analytical outcomes. Although *Fukomys* mole-rats were able to distinguish between urinary odours of familiar siblings, they did not differentiate those odours from an unknown stranger, an odour, whose difference to own siblings should be more pronounced than between siblings (chapter IV.1.4.1). The present study thus implies that the similarity between siblings (verified by chemical analytical findings) could not be detected on the basis of urinary odours by mole rats. Instead, they detected tiny differences between siblings, between familiar and even between stranger individuals, whose urinary odourous profile, the template, could not have been learned through former encounters.

Moreover, chemically proved similarities of urinary composition within male siblings and their respective fathers (kings), do not support the hypothesis of odour-genes covariance (chapter IV.1.1.1; the closer individuals' genetic background the closer their odour). Concordantly, behavioural findings suggest that genetic covariance is not reflected in urinary odour: neither the similarity between siblings nor the differences between two (unrelated or sibling) strangers were detected on the basis of this source of odour. Additionally to findings discussed above (chapter IV.1.4.1), showing that urine odour does not provide enough information about individuals' identity in mole-rats, the chemical analyses support low olfactory importance of urine in this context. This can be supported by chemical similarity of urinary volatiles between individuals of all reproductive categories in males and also the similarity within the same reproductive category of females (where differences were found only between queens and daughters, see below).

Reproductive status of females (differences between Q and nrf)

Contrary to findings in respect to individuality and family urinary volatiles, the reproductive status of females had significant influence on their urinary chemical composition, both qualitatively and quantitatively. One component (peak 3), 4-nonanone, clearly could be proved to be a typical compound in queens (tab. IV.3.4, fig. IV.3.7a). A further component (peak 33) was found to be significantly higher in queens and further three compounds (peak 36, 37 and 40) were conspicuously higher in queens compared to

non-reproductive females (cf., tab. IV.3.4 and 3.5; fig. IV.3.9, fig. IV.3.8). The reproductive status thus is reflected in urinary volatiles and allows us to distinguish queens from non-reproductive females by their chemical urinary metabolites. The question however is, whether this finding is of any relevance to males. It is imaginable that queens which are already sexually and endocrinological primed (as stated above; cf., chapter IV.2.2.3) through repeated copulations with the king, could be of more interest even for strange male intruders, because of expected higher and faster reproductive success compared to inexperienced and thus not primed daughters. Altered metabolism and therefore urinary excretions were already found to be reflected in hormonal profiles by reproductive activity. Sexually active queens display in their urine extraordinarily higher estradiol and progesterone concentrations compared to their sexually inactive daughters (Hagemeyer et al. 2009). The chemical analytical findings of the present study support those findings and demonstrate further differences in the urine composition, additionally to steroidal compounds, between both female categories.

In addition, concurrent behavioural data underline the chemical analytical findings, since especially non-reproductive males were able (and highly motivated) to prefer queens compared to non-reproductive females on the basis of urinary odours in preference tests (cf., chapter IV.1.4.2). On the basis of ano-genital odours, the behavioural preference test delivered even stronger results, indicating a clear preference of non-reproductive males for queens, thus supporting the idea of even more meaningful metabolites to *Fukomys* mole-rats which are obviously not limited to urine. My chemical analytical results, i.e. a different urinary volatile pattern, in respect to the female reproductive status, thus led me to the conclusion of certain relevance of urine for mole-rats as a chemical signal, although the ano-genital odours seems to encode compounds of higher significance to the individuals.

The finding that some of the urinary components, which are found to be of higher concentrations in queens, are affected by carrot and/or potato diet, demonstrates the need of further studies to clarify the relevance and the influence of such metabolic dynamics for *Fukomys* mole-rats. Supporting data could be achieved, for instance, by looking for the presence of 4-nonanone in the urine of a queen, first during normal food intake and subsequently during carrot- and potato diet respectively, since this study shows the influence of diet on urinary volatiles from family samples, but not from individual samples as a highly interesting but still fragmentary result.

The identified ketone 4-nonanone is a specific queen urinary volatile compound which plays a putative role as a sexual attractant for males. A related ketone, 2-nonanone, was described to act as a musth-related and thus chemosignalling urinary component in male African elephants (*Loxodonta africana*) (Rasmussen & Wittemyer 2002), whereas 4-nonanone

has not been described yet to play a role as chemical signal. This component was, however, identified in higher concentrations in the urine of male hamster (*Phodophus campbelli* and *P. sungorus*) in two chemo-analytical (one methodological) studies of Soini et al. (2005 a and b), but unfortunately no further behavioural functions or meaning to females were discussed there. Since the current study demonstrated a correlation of the urinary compound 4-nonanone to the reproductive females (the queens), a chemosignalling function of this ketone seems to be obvious, i.e. a "queen identifying" component. Further studies (involving a larger number of queens) are needed to prove this hypothesis. Testing the presence (or absence) of this ketone in the urine, followed by preference test of males with the pure compound (per analysis), solved in water should complement our hitherto insight. It is imaginable, that the proved chemical urinary differences between queens and their daughters will culminate in detection of more pronounced differences in ano-genital odours between both female categories.

The other four compounds, which were not yet identified in detail by mass spectra, differ between queens and their daughters in concentrations (cf., fig. IV.3.8). Peak 33 was significantly higher in queens. Mass spectral analysis demonstrated that this component is similar to quinoline. Zhang et al. (2005) described this compound to be male – specific in ferrets (*Mustela furo*). Thus, it probably plays a comparable role in queens here, as much as the compound at peak 40, which seems to be related to the terpene farnesene (cf., tab. IV.3.5). The role of this compound has been described in the house mouse (*M. m. domesticus*), e.g. to signal dominance in males (Novotny et al. 1990) or inducing estrus in females (Ma et al. 1999). Since concentrations of those three components differed significantly also between queens compared to other family members (including K, nrm and nrf), above findings are supported and demonstrate queen-specific differences of metabolic excretions (cf., fig. IV.3.10). Note that the high standard deviation results from the small number of queens. Further analytical studies, with an larger sample size (of queens), thus should result in more pronounced differences. It is probable, that not a single compound, like 4-nonanone, but rather a chemical mixture signifies the reproductive status of the queen, which should be more pronounced in ano-genital excretions, because the behavioural outcome was stronger.

Surprisingly, one daughter of family two (nrf E) showed a peak-pattern, resembling that of queens (cf., fig. IV.3.9d). Since she was two years and three month old, i.e. younger than some of the other non-reproductive females (ranging from two to seven years), this effect could not have been caused by aging and/or maturation. Also puberty, i.e. the first estrus, needs to be primed by repeated copulation (cf., chapter IV.2.2.3) and therefore cannot provide a satisfying explanation. This female moreover never had contact to strange males.

Anyhow, the queen-specific peak, 4-nonanone, could not be detected. Moreover, peak 17 at RT = 38.0 min was found, a peak generally missing in queens. Both peaks, 4-nonanone and peak 17, thus rather support the finding of a queen-specific urinary volatile profile.

The component 4-nonanone was clearly identified in this study by its mass spectra whilst the two other described components (quinoline and farnesene) need to be confirmed by following analysis.

Differences in urinary volatiles between kings and non-reproductive males

Contrary to findings in females, kings cannot be distinguished from non-reproductive males (sons) by means of their urinary composition, since their TIC-pattern of GC-MS are very similar and differ in single peaks even by chance (cf., fig. IV.3.7b). It is thus not possible to differentiate kings from their sons on the basis of urinary volatiles. This result should not be astonishing assuming a constant physiological, sexual, and reproductive readiness of males even without experience. Therefore, it is imaginable that in the scenario of an encounter between a strange male and a female, composing a potential reproductive pair, simply do not need such differing mechanism between males (in contrast to female reproductive categories as argued above). Even intruding females (which have never been observed or described in the literature) would not need to differentiate a strange king from a son, because both can represent a potential reproductive partner.

Differences in urinary volatiles between females and males

Results from males compared to females indicate strong qualitative similarity within non-reproductive females, queens and non-reproductive males respectively, but high differences between both sexes and within both female reproductive categories, i.e. between queens and their daughters. ß-caryophyllene (peak 24 at RT = 39.7 min) could be identified (from mass spectra and co-injection) as one male-typical component, since its %-area and its absolute concentrations were significantly higher in males than in females (cf., fig. IV.3.6a, fig. IV.3.7a,b). Interestingly, ß-caryophyllene was not affected by potato- or carrot diet. *Fukomys* mole-rats thus seem to metabolize in nature this component probably irrespective of its availability in food. This suggests its high relevance as a signal for mole-rats.

The sesquiterpene ß-caryophyllene has already been proved by Achiraman & Archunan (2006) to be an estrus-specific urinary chemosignal in the female house mouse (*M. m. musculus*). The authors stated, that endocrine changes influence variations in the urinary composition. Several studies proved pheromonal effects of this component mainly in insects, for instance in the multicoloured Asian Lady Beetle (*Garnibua axyridis*): In a whole-air sampling method ß-caryophyllene was found to be a gender specific component, emitted only by females (Brown et al. 2006). In the Colorado potato beetle (Khalilova et al. 1998),

the green lacewing (Flint et al. 1979), carrot fly (Guerin et al. 1983), and the damson hop aphid ß-caryophyllene serves as an attractant (Campbell et al. 1993). These examples of multiple studies on ß-caryophyllene in species of differing taxonomy points out the broad range of action as an olfactory chemosignal, either within or between species. The present study additionally points out a probable sex specific function in *Fukomys* mole-rats, comparable to the house mouse. Contrary to the study of Achiraman & Archunan (2006), however, in mole-rats it seems to be a male-specific urinary chemosignal, independent from the status of males, i.e. king or non-reproductive son.

Since it was proved here, that ß-caryophyllene cannot be correlated to diet, it would be of high interest, whether this component is metabolized in correlation to the endocrine status, e.g. to testosterone levels. It is well known that terpenes are sex-dependent in animals. The sesquiterpenes *E,E*-α- and *E*-β-farnesene for instance, are absent or scarce in immature and castrated male house mouse (Harvey et al. 1989) and have seldom been described in females so far (e.g. for low production in females, Zhang et al. 2007). Furthermore, both terpenoids are related to steroid intermediates (Novotny et al. 1999b), and are dependent on the preputial gland which is under androgen control (Harvey et al.1989, Zhang et al. 2007). Thus, it is assumed here that also the sesquiterpene ß-caryophyllene could be dependent on androgenes such as testosterone.

Kramer (2006) did, however, not find differences in the testosterone concentrations between kings and non-reproductive male *Fukomys* mole-rats, which fits well to the findings of the present study, since the ß-caryophyllene levels of urinary volatiles between both male categories were comparable. Strikingly, reproductively active females (queens), show significantly higher testosterone concentrations then all other categories (nrf, nrm and kings, Kramer 2006). However, since the ß-caryophyllene concentrations are comparable between queens and non-reproductive females (fig. IV.3.7) we can conclude that ß-caryophyllene is not a testosterone-controlled compound. Moreover, since also further steroids, e.g. estradiol and progesterone concentrations are higher in queens than in reproductively quiescent females (chapter IV.2.1.3.2), it is probable that ß-caryophyllene generally is steroid-independent. Thus, despite interesting differences between males and females regarding ß-caryophyllene, our knowledge about the metabolic pathway of that compound remains enigmatic and is far from complete.

The second male-specific component (peak 23) was detected at RT = 39.5 min. Females had noticeably lower values of it than males. Mass spectra identified this compound to be 2-methoxy-4-vinylphenol but since this compound was not confirmed by co-injection, its identity remains tentative. This compound has not been described yet in the context of pheromonal activity.

Four further female-specific components (peak 5, 6, 44 and 45) need to be dealed with caution. The %-areas of all those compounds were very low, indicating relatively low amounts. Moreover, they were missing also in some females (queens and/or non-reproductive females likewise) and present in one non-reproductive male. Thus, in sum the male-specific profile is characterized mainly by high amount of ß-caryophyllene and 2-methoxy-4-vinylphenol and low contribution to peak %-areas of the other 50 peaks in the overall profile compared to non-reproductive females and especially to queens (fig. IV.3.6).

Qualitative analysis (mass spectra)

Altogether five compounds were definitely identified by mass spectra (cf., chapter IV.3.3.8), of which one belongs to the compound class of sesquiterpenes (isoprenoids) and one to a cyclic terpene, both typical constituents of essential oil. Moreover, two compounds belong to ketones and one compound belongs to alcohols. Five further compounds identified by mass spectra need to be verified by further analysis, since their identity remains tentative so far. Two of them belong to the compound class of esters, one to alcohol and one is a ketone. Those compound classes, terpenes, alcohols, aldehydes, esters and ketones, are well known to be of olfactory relevance in several species and/or were often found to be pheromone-active (e.g. Albone 1984, Soini et al. 2005a, Zhang et al. 2005, Zhang et al. 2008). Most of the compounds found in this study are non-polar, for instance 1-hexadecanol or octadecanoicacidbutylester (with their long hydrocarbon chain and therefore a lipophilic substance), whilst 2-methoxy-vinylphenol is a polar and water soluble substance. Lipophilic compounds are capable to be ligands of the hydrophobic pocket of MUPs (major urinary proteins), but this will be the subject of the next chapter and will be discussed in more detail therein. Some of the described compounds seem to be of certain relevance for *Fukomys* mole-rats and thus are described in more detail below.

Genus-specific: Both, hexadecanoicacidbutylester, peak 50 at RT = 44.7 min and octadecanoicacidbutylester, peak 51 at RT = 45.7 min (cf., fig. IV.3.7) were most constantly found in comparable amounts in every individual, regardless of sex, family or reproductive status, thus probably constituting the genus-specific odourous mixture of urine in *Fukomys* mole-rats. Both compounds, however, have to be confirmed by mass spectral identification, since they were identified with a match below 900 (this study). Four further compounds seem to contribute to the genus specific odour, namely 1,3,8-p-menthatriene and (E)-2-octenal of which the latter remains to be verified by mass spectra as well, but both of them were found in all individuals in similar concentrations.

The component 1,3,8-p-menthatriene belongs to the class of terpenes and is a typical fragrance of some fruits and herbs, e.g. in mango (Pino et al. 2005) and also in parsley (Simon & Quinn 1988). It is obvious that it is metabolized dependently on access to food

and to food preferences. Indeed, this component was influenced by diet, both potato or carrot diet (cf., tab.3.3 and fig. IV.3.4). Its concentration decreased significantly, especially when the animals were kept on potato diet. Interestingly, this component was actually proved in low amounts in carrots (Weissteiner & Schütz 2006). To date, however, this component has not been found in potatoes, thus probably explaining the larger decrease in urine of *Fukomys* mole-rats kept on potato diet only. Notably, 1,3,8-p-menthatriene (peak 6) was, on the other hand, not well separated from 4-methylphenol in the urinary mixture of families by gas chromatography. Therefore, it is hitherto not quite clear, whether the diet influenced 1,3,8-p-menthatriene or 4-methylphenol or both. The olfactory or pheromonal relevance of 1,3,8-p-menthatriene for animals have not been described yet. However, it is imaginable that it serves as a kairomone (food odour) of plants, whilst the function in the urine of *Fukomys* mole-rats, besides a probable contribution to genus-specific odour, remains unclear so far. Acetophenone (peak 9 at RT = 34.3/4 min) and 4-methylphenol (peak 8 at RT = 33.7 min), were found also in high levels in all the individuals under study, yet kings seem to produce less of both compounds compared to other individuals (fig. IV.3.7).

Interestingly, the compound 4-methylphenol, in *Fukomys* mole-rats presumably also contributing to the genus-specific compound, is present in Asian and African elephants (*Elaphas maximus* and *Loxodonta africana*) in the secret of the temporal gland (Rassmussen & Perrin 1999). Strikingly, this compound was also detected in human sweat as an attractant, triggering a strong response of a AgOr1 protein (a putative odourant receptor) of *Anopheles gambiae* (Hallem et al. 2004).

Acetophenone is the simplest aromatic ketone and was recently decribed in the western pine beetle playing a roole for pest-control as an anti-attractant (Erbilgin et al. 2007). More interestingly, however, a related compound, *o*-aminoacetophenone, known as a strong "weasel smell" compound, was proved by Zhang et al. (2005) in the anal glands of ferrets (*Mustela furo*). Moreover, acetophenone was also identified, but unfortunately not discussed by Zhang et al. (2005). In honeybees (*Apis mellifera*), *o*-aminoacetophenone was additionally found in feces of virgin queens acting on agonistic interactions between those queens and workers (Katzav-Gozansky 2006). Furthermore, the authors decribed it as a repellent for workers. Although *Fukomys* mole-rats are also eusocial like honeybees, one should not assume similar interactions to occur also between queens and their non-reproductive daughters of mole-rats. Indeed, it could be demonstrated that acetophenone is present in all the social and reproductive categories. This compound thus probably just contributes to the genus-specific odourous "bouquet" of *Fukomys* mole-rats. All the different species, sharing the same compound (in different contexts), accentuate functional flexibility of this and other compounds, acting in species even in different taxonomic classes.

Most of the presumed genus-specific components found here are independent from the carrot or potato diet (except of 1,3,8-p-menthatriene and 4-methylphenol), thus probably indicating high relevance for the animals.

Queen-specific: The compound 4-nonanone has been already discussed above in detail as a queen-specific compound.

Male-specific: Also ß-caryophyllene was proved to be related to sex and not to reproductive activity per se (cf., the discussion above). Besides its proposed sex-related function, it may also contribute to the genus-specific odour of *Fukomys* mole-rats, since, despite the lower concentrations found in females, it was found in all the individuals.

MUP-related: Strikingly, 1-hexadecanol (peak 47, RT = 43.3/4 min), a compound found in this study solely in non-reproductive females, was identified in the house mouse by Zhang et al. (2007) to be, however, male-specific being associated with the preputial gland. Moreover, it was shown, that it is involved as a ligand of MUPs (major urinary proteins), as will be outlined in the last chapter. Without further analytical work its identification remains tentative, but nevertheless this substance should be under further focus with respect to its gender-specifity.

The interpretation of the results presented in this study is difficult because of, i.a. the identified 51 peaks of individual urine were detectable in every individual, but were not always well separated by gas chromatography or identified equally by mass spectrometry (many of them remain tentatively identified or await further identification). The retention time of 1,3,8-p-menthatriene and 4-methylphenone, for instance, showed a strong overlap in the urine-analysis of families (both constituting peak 6; cf., chapter IV.3.1.3.8 and the discussion above). Optimizing and narrowing several parameters of the gas chromatograph, e.g. temperature program, gas flow rate and the spit/splitless time, should overcome successively the problem of every hitherto suboptimal separated peak. In addition, those effects of overlap probably mask some components, which might be more important to *Fukomys* mole-rats. Moreover, it remains to be solved by ongoing studies, whether the compounds analysed by the three-component 50/30 µl DVB/CAR^{TM}/PDMS^{TM}Stable Flex^{TM}-fibre, mainly extracting semi-volatile and volatile compounds, are of any pheromonal relevance for *Fukomys*-mole-rats or whether some other, hitherto not extracted substances may be probably even more important for mole-rats. Compounds with different physical characteristics, e.g. not volatile or highly volatile, remain thus enigmatic. For several reasons, however, in the subterranean environment, highly volatile compounds most probably do not

work, e.g. they would be mixed uncontrolled to an indefinable odour, whose releaser would remain unknown to the recipient.

To sum up, with the newly developed, very effective emerging method of HS-SPME-GC-MS it was possible to take a closer look upon the behavioural based data of individual and family odours of *Fukomys* mole-rats. Out of six different families, the urinary volatile profiles of five those families were similar with respect to the retention time and mass spectra, since all the families exhibited 41 different peaks. Moreover, 51 individual urinary components were detected. It could be demonstrated, that the carrot as well as the potato diet influenced the urinary pattern. Interindividual variations were found to depend on sex (male or female) and reproductive status (Q and nrf) and not on individuality per se. The urinary chemical composition of individuals within each particular reproductive category was quite similar. In all of the three tested queens, a small queen-specific compound, 4-nonanone, could be verified, whilst it was absent in every non-reproductive female. Furthermore, the urinary profiles of queens differed from their non-reproductive daughters by further four peaks, which were more abundant. Kings, however, could not be distinguished on the basis of their urinary volatile pattern from non-reproductive sons. Males can be distinguished from females on the basis of urinary pattern by the higher amounts of ß-caryophyllene. Five of those compounds were identified by mass spectra and further five compounds were tentatively identified by this means, some of them probably contributing to the genus-specific urinary odour of *Fukomys* mole-rats. The identified compound classes belong to aldehydes, ketones, alcohols and terpenes, all of those classes were described in the literature to have pheromonal and/or olfactory impact in a wide range of species. The importance of all these findings for the olfaction of *Fukomys* mole-rats largely remains enigmatic yet. Broadening the analytical investigation to body secretions, e.g. ano-genital odour, should expand our hitherto knowledge.

Recently, a further solvent-free sampling method, stir bar absorptive extraction technique (SBSE), was shown to be even more effective than SPME (Soini et al. 2005b, Baltussen et al. 2002). Baltussen and his colleagues demonstrated (2002) in their study that in contrast to SPME, restricted to extract apolar compounds, with the method of SBSE apolar as well as polar compounds could be extracted. Consequently, besides broadening our investigations to further urine excretions, also using SBSE should elucidate some hitherto "hidden" urinary compounds, probably more important for *Fukomys* mole-rats, which is, however, a question of research funding. Body secretions, which are probably semi-volatile or non-volatile, need, moreover to be extracted by developing a SPME-method. In this way, very likely, relevant compounds for *Fukomys* mole-rats will be revealed.

IV.4 LIPOCALINES (MUP´S & APHRODISIN) - PROTEOMICS

IV.4.1 INTRODUCTION

Behavioural evidence clearly shows that rodents depend on odourous secretions and excretions to communicate everything of social importance: their individual identity, sex, age, health, reproductive and social status, etc. (as stated above and in parts demonstrated in the previous chapters). The processes underlying transmission of these complex semiochemicals from sender to receiver, however, remain enigmatic. It has been suggested that variations in major urinary proteins (MUPs) with or without their bound ligands are the primary (Beynon & Hurst 2003 and 2004) or even exclusive (Hurst et al. 2005, Cheetham et al. 2007, Sherborne et al. 2007) conduits of chemosignals because of their presumed role in conveying individual identity, species, and dominance status in male house mice (Hurst et al. 2001, Beynon et al. 2007). Studies on MUPs in wild rodents are scarce, but there is abundant behavioural evidence (reviewed e.g. in Todrank & Heth 2003) of adaptive responses – such as discriminations and preferences – to the individual genotype (along with information about population and species) and the changing biological states expressed in odours.

These patterns of responses were found consistently across diverse rodent species with different social structures and originating from different continents. Investigating the prevalence of MUPs in urine of these rodents could shed light on chemosignal transmission processes.

Major urinary proteins are expressed in the liver, filtered by the kidneys, and excreted through urine, at quantities constituting up to 99% of the protein content in adult male house mice (Humphries et al. 1999). Proteins in the MUPs family belong to the

Fig. IV.4.1: Three dimentional fold of the MUP with its eight β-strands (in front violet, green, yellow, blue; behind bright blue, red, black and pink) arranged in a barrel like configuration. The eight strands create a central calyx (cavity bindig side) that binds volatile ligands (green cyclic molecule). Adapted from Overhiser (1999) and URL1.

lipocalin superfamily (Flower 1996, Akerstrom et al. 2000) and are characterized by a three-dimensional fold composed of an archetypical eight stranded β–barrel and an α–helix located in the C-terminal region. The β-strands form a hydrophobic pocket in which small, volatile and non-polar molecules can be bound for release into the environment (Lücke et al. 1999, Cavaggioni & Mucignat-Caretta 2000). Figure IV. 4.1 shows the three dimensional fold of the MUP illustrating bound ligand in the central calyx. MUPs have mass ranges of approximately 19 kDa (Robertson et al. 2007). Another member of the lipocalin superfamily with a mass of approximately 17 kDa (Henzel et al. 1988), aphrodisin, was identified in the vaginal discharge of hamsters (Singer et al. 1987, Henzel et al. 1988, Singer 1991, Mägert et al. 1995) and serves as a pheromone carrier in hamsters (Jang et al. 1995 and 2001, Briand et al 2004). Aphrodisin is a lipocaline, whose ß-barrel structure is well suited to bind small hydrophobic molecules (Briand et al. 2000a, Vincent et al. 2001).

Studies of MUPs have typically targeted few strains of laboratory mice, e.g. C57BL/6J or BALB/cJ (i.a. Berger & Szoka 1981, Bennett et al. 1982, Mucignat-Caretta et al. 1995, Novotny et al. 1999b, Robertson et al.1996, Marie et al. 2001, Cavaggioni et al. 2003, Nevison et al. 2003, Beynon & Hurst 2004, Armstrong et al. 2005) and rats (MacInnes et al. 1986, Bocskei et al. 1991, Lehman-McKeeman at al. 1998, McFadyen & Locke 2000, Mucignat-Caretta et al. 2006), but just few studies have been conducted on wild house mice (e.g. Hurst et al. 2001, Beynon et al. 2002, Robertson et al. 2007, Stopkova et al. 2007). Ligand binding and releasing by MUPs is thought to protect volatile odourants from degradation once urine is deposited and extend the longevity of the chemosignal through the slow release mechanism (Robertson et al. 1993, Novotny et al. 1999b).

Because initial studies showed high polymorphism in MUPs (Robertson et al. 1996), it was concluded that variants in proportions of particular MUPs contribute inherently to signal individuality in urine scent marks (Hurst et al. 2001). Few studies supported direct involvement of MUPs in conveying identity, species, and biological state (e.g. Mucignat-Caretta et al. 1995, Beynon et al. 2002, Nevison et al. 2003, Martínez-Ricós et al. 2007) while other studies suggested an indirect role of MUPs through active ligand binding or ligand/MUP-complexes (e.g. Novotny et al. 1999a and 1999b, Peele et al. 2003, Robertson et al. 2007).

Comparative studies of the prevalence and modulation of MUPs in further rodent species, like in *Fukomys* mole-rats, in which the importance of semiochemicals has been demonstrated behaviourally (cf., chapters IV.1.4.1 and IV.1.4.2), could enlighten questions about the general function of MUPs in chemical communication. Recent studies indicate the presence of MUP-like proteins (identified by *de novo* peptide sequencing as

aphrodisin) also in urine of hamsters (*Phodopus roborovskii*) and bank voles (*Myodes glareolus*), but at much lower concentrations (Beynon et al. 2007). It is therefore questionable whether generalizations to other groups of rodents or mammals are possible. In this study, urine samples from species belonging to three different subterranean genera representing different rodent families and diverse social systems were examined. Hitherto unknown urinary proteins of each genus were analysed and compared with respect to their concentrations by the Bradford test followed by 2D-PAGE (*two dimensional-polyacrylamid-gel-electrophorsis*), constituting of *isoelectric focusing*, IEF (first dimension), and *sodium dodecylsulfate-polyacrylamide-gel-electrophoresis,* SDS-PAGE (second dimension). With the 2D-PAGE analysis, proteins can be separated according to their charge by IEF depending on their pI (isoelectric point), whilst SDS-PAGE is a proper method to separate them according to their mass in kDa (Hamdan & Righetti 2005). Since no proteomic analytical investigation of urine of *Fukomys* mole-rats (nor in any other subterranean rodent) was performed so far, the methodic protocol for the laboratory house mouse was adjusted for the *Fukomys* mole-rats here, in order to find any signs of MUP-related lipocaline-like proteins. Those proteins (approximately 20 kDa dots, with an isoelectric point ranging between 4-5 pI) were subsequently identified after tryptic digestion by MALDI-TOF/TOF-MS/MS (*matrix-assisted-laser-desorption-ionisation – time-of-flight – tandem – mass-spectrometry) de novo* sequencing.

Commonly, after (tryptic) digestion of the protein, the pattern of the generated peptide fractions (fractionated e.g. by *high pressure liquid chromatography*, HPLC or by TOF, *time of flight*) constitutes the *peptide mass fingerprint* (PMF) of the cleaved protein, detected by their mass dependent time of flight generating mass spectrum (MALDI-MS) (cf., Demine & Walden 2004). Additionally, those peptides need to be further fractionated into positive monocharged N-terminal and C-terminal amino-acids (labeled in a mass spectrum as a, b or c-ion series and x, y or z-ion series) by collision of gas (*collision induced decay,* CID), delivering mass spectra for each fractionated peptide (precursor). By means of those spectra, the so called *de novo* analysis (i.e. deducing the peptide sequences directly from the mass spectra; Demine & Walden 2004) follows by matching the obtained amino-acid sequence to a reference sequence (*local alignment*), lodged in database of the National Centre for Biotechnology Information (NCBI) by BLAST (*basic local alignment search tool* (Altschul & Lipman 1990, Armstrong et al. 2005, Hamdan & Righetti 2005, Haupt 2006). Nesvizhskii (2006) described the advantage of the *de novo* sequence in that it allows the identification of peptides, whose exact sequence is not present in the searched database. On the other hand, the author explains that this methodological approach requires high-quality MS/MS spectra, whose deduced sequence need to be matched e.g. using

BLAST, against sequences of known proteins in the database. For my purpose this, however, was not expected to be a disadvantage, since the target lipocaline-like sequences are already well investigated und therefore their sequences are lodged in databases of e.g. NCBI or SWISS-Prot.

The first group of studied animals included the *Fukomys* mole-rats (*F. anselli*, *F. kafuensis*, and their hybrids*)*. The second group included four species of subterranean blind mole-rats, *Spalax ehrenbergi* superspecies (*S. galili*, *S. golani*, *S. carmeli,* and *S. judaei*), which are solitary and territorial (Nevo et al. 2001). The third studied genus was the social coruro, *Spalacopus cyanus*, which is polygenous (Begall 1999). These genera originate from three continents: Africa (*Fukomys*), Eurasia (*Spalax*), and South America (*Spalacopus*) (tab. IV.4.1).

Animals of all these species have previously been shown to respond differentially to urine and to various secretions of conspecifics according to individual identity, reproductive readiness or social status. Odours in urine of *Spalax* blind mole-rats are important for individual, population, and species discrimination (Heth et al. 1996a, Todrank & Heth 1996, Heth & Todrank 2000). Blind mole-rats also respond differentially to urine odours depending on the season (breeding or non-breeding) in which samples were collected and the season in which subjects were tested (Heth et al. 1996b). By means of the behavioural and the described chemo-analytical investigations of the current study, the importance of chemical communication was demonstrated within families of *Fukomys* mole-rats (cf., chapter IV.1 and IV.3; Heth et al. 2002) and coruros (Hagemeyer & Begall 2006).

Thus, odours of urine and/or ano-genital secretions indicating individual identity and reproductive status clearly are important in behavioural responses in all the species under study here (except of urine for individual discrimination in *Fukomys* mole-rats; cf., chapters IV.1 and IV.3). It is not clear, however, whether urinary proteins are involved in chemical communication in subterranean rodents. The goal of this part of the thesis was to provide biochemical analysis of urine from these subterranean rodents paying particular attention to such proteins.

IV.4.2 MATERIAL AND METHODS

IV.4.2.1 Animals

The urine of 63 animals representing three genera of subterranean rodents were tested in comparison with urine of the house mouse (tab. IV.4.1).

Coruros and *Fukomys* mole-rats (either wild-captured or laboratory-bred) were kept at the Department of General Zoology in Essen (Germany), under the conditions, described in detail in chapter IV.1.2.1. The blind mole-rats were captured in the field and kept in the Institute of Evolution at the University of Haifa[7]. Each of the four species was kept in a separate room under a 14:10 hour light: dark cycle with constant temperature (21 ± 1° C) and relative humidity (70 ± 5%). The animals were kept individually in plastic cages (30 x 40 x 25 cm) and were provided carrots and potatoes *ad libitum*. Subterranean rodents do not drink free water but obtain liquids from their food.

The laboratory mice (C57BL/6), used as a reference species for the total protein assay and for SDS-PAGE and 2D-PAGE as a standard in the specific protein analysis, were kept under standard laboratory conditions at the Chair of Zoology of the Charles University in Prague. They were provided water and commercial mouse pellets *ad libitum*.

[7] Urine samples of blind mole-rats were friendly provided for analysis by Professor Dr. Giora Heth & Dr. Josephine Todrank (Institute of Evolution at the University of Haifa).

Tab. IV.4.1: Sample sizes, sex, age class, continent (country) of origin, and number of specimens with MUP/lipocalin-bands in SDS-PAGE. Q = queen; K = king.

	sample size	sex ♂	sex ♀	age class	continent (country)	specimens with bands
F. anselli (Bathyergidae)	12	2	5	2K,3Q 7 juveniles	Africa (Zambia)	12
F. kafuensis (Bathyergidae)	6	1	3	1K,1Q, 4 juveniles	Africa (Zambia)	6
F. anselli x *F. kafuensis*	11	4	1	3K, 3Q, 5 juveniles	Africa (Zambia)	11
Spalacopus cyanus (Octodontidae)	8	6	2	adults	South America (Chile)	0
Spalax galili (Spalacidae)	5	3	2	adults	Eurasia (Israel)	0
S. golani	2	1	1	adults	Eurasia (Israel)	0
S. carmeli (Spalacidae)	8	6	2	adults	Eurasia (Israel)	2 ♂
S. judaei (Spalacidae)	10	6	4	adults	Eurasia (Israel)	0
C57BL/6(Muridae)	1		1	adult	Laboratory	1
Total	63	32	21			

IV.4.2.2 Urine collection

Urine collection of *Fukomys* mole-rats is described in detail in chapter IV.1. Since coruros, however, seldom urinated when they were picked up, they were put into a clean bucket. When they urinated there, the urine was immediately transferred by a pipette into an Eppendorf-tube. Urine samples were centrifuged at 8,000 rpm for 8 seconds, and the supernatant was transferred by pipette into a sterile Eppendorf tube. Following this procedure, the urine samples were frozen at -80° C until analysis.

Urine from blind mole-rats was collected by picking up the animal and massaging the hind quarters until it urinated into a sterile plastic cup (from which the urine was transferred to sterile Eppendorf tubes and frozen at -80° C for shipment). Urine from mice was obtained by gentle abdominal massage until urination into a 1.5 ml Eppendorf-tube and frozen immediately at -20° C.

IV.4.2.3 Bradford test and creatinine determination

Total protein concentrations in urine were measured by the Bradford method (Bradford 1976) using bovine serum albumin with known concentrations to creatinine (Cr) standard curve ($R^2 > 0.98$) (Quick Start™ Bradford Protein Assay; Bio-Rad Laboratories, Hercules, CA, USA).

The total protein concentration of each sample was corrected for creatinine concentration (Snyman et al. 2006). For each sample, the creatinine concentration was determined using the Jaffe reaction (Folin 1914). The freshly prepared picric reagent (4 units sodium hydroxide, 1 unit picric acid) was incubated for 15 min at room temperature in darkness (Bio Vender Lab., Brno, Czech Republic). 50 µl of standard, water (blank) or sample was added to a 1 ml cuvette, mixed and measured spectrophotometrically at absorbance of 492 nm after 30 and 90 seconds, respectively.
The following formula to calculate the molar creatinine concentrations was used:

$$C_{creatinine} = \frac{C_{st} \cdot (\Delta A_{sample} - \Delta A_{blank})}{\Delta A_{standard} - \Delta A_{blank}} [\mu mol / l]$$

C_{st} = concentration of standard (337 µmol/l, 0.038 mg/ml), A = absorbance, and delta A = (A after 90s – A after 30s). All statistical analyses were performed using SPSS (version 14) software.

IV.4.2.4 SDS-PAGE *(sodium dodecylsulfate-polyacrylamide-gel-electrophoresis)*

Urinary proteins were precipitated by pipetting 100 µl urine into 500 µl acetone and centrifuged at 14,000 rpm for 5 min. Then, the samples were dissolved into 15 µl sample buffer (3.55 ml H_2O, 1.25 ml 0.5 M Tris/HCl (pH 6.8), 2.5 ml glycerol, 2.0 ml 10 % SDS, 0.2 ml 0.5 % bromphenol blue). A modified protocol (Laemmli, 1970) was used: samples were applied to 4-15% and 4-20% SDS-PAGE discontinuous gradient gels (precast-gel, BioRad Laboratories, Hercules, CA, USA) and run at 50 V for 20 min followed by 80 V for 120 min using a mini-gel apparatus (Mini-PROTEAN II Electrophoresis System, BioRad Laboratories, Hercules, CA, USA). The gels were stained with Coomassie® G-250 (SimpleBlue™ Safe Stain, Invitrogen Life Technologies, Paisles, UK) for 1 hour and destained with deionized water. Prestained molecular weight standards (Sigma-7-Ladder, BioRad Laboratories, Hercules, CA, USA) were run with each set of samples. As an internal MUP-standard, 1 µl natural urine was used (i.e. not precipitated with acetone before SDS-page) from a male laboratory mouse (C57BL/6) that was loaded into 10 µl sample buffer. Images were collected with a GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, CA, USA).

The urine of all the studied animals was tested by SDS-PAGE in order to find references for the existence of proteins in the mass range of MUPs (or lipocalins) (tab. IV.4.1). Samples indicating proteins were used for 2D-PAGE (see below).

IV.4.2.5 2D-PAGE (*two dimensional-polyacrylamid-gel-electrophoresis*)

IEF (Isoelectric focusing)

Isoelectric focusing was performed for the most appropriate four specimens according to SDS-PAGE results (two males, one non-reproductive female und one queen of *Fukomys* mole-rats and two males of *Spalax*) using the Biorad Protean® IEF Cell. It was necessary to concentrate the urine of *Fukomys* and *Spalax* before further treatment. First, 800 µl of urine was centrifuged at 15,000 rpm for 10 min. Urine samples were fractionated in 300-400 µl aliquots, which were transferred by a pipette into a Millipore-column (5 kDa, Ultrafree-MC) and spun at 15,000 rpm for 20 min. The samples were washed and centrifuged again at the same speed for 20 min several times with a 1:4 times diluted PBS-buffer until the stock solution was reduced to a volume of 50-100 µl. From this stock solution, 15 µl was precipitated into 250 µl acetone. Then, 7 cm IPG-strips (pH range 3.9-5.1 and 3-10, BioRad Laboratories, Hercules, CA, USA) were dissolved in 120 µl rehydration buffer (4.2 g urea, 1.5 g thiourea, 0.2 g CHAPS, 200 µl of 1M DTT, 10 µl of 0.5% bromphenol blue, 1 ml of 100x ampholytes pH 3-10; Bio-Lyte, BioRad Laboratories, Hercules, CA, USA, in 10 ml distilled water). The strips were allowed to rehydrate passively overnight at 4° C. The program for focusing was as follows: One hour at 50 V, 25 µA per strip at 15° C and a voltage limit of 4000 V. Focusing was finished after 16,000 Vh. Equilibration was done with 4 ml of two SDS equilibration buffers for 15 min. The first buffer contained 6M urea, 0.375M tris pH 8.8, 2% SDS, 20% gycerol, 2.5% DDT. The second buffer had the same components, but DDT was replaced by 2.5% iodoacetamide. Then, the IPG strip was placed onto a second Dimension SDS-PAGE. With each set of samples an 8 µl sample molecular weight standard (Sigma-7-Ladder, BioRad Laboratories, Hercules, CA, USA) ran simultaneously. The most concentrated *Fukomys* samples came from two male subjects (numbers 11 and 14) and were used for 2D-PAGE (cf., fig. IV.4.3). The only samples from *Spalax* that contained detectable amounts of protein were samples 5 and 7 (cf., fig. IV.4.4). As the tested *Spalacopus cyanus* samples did not show any bands in sodium dodecyl sulfate-polyacrylamide (SDS-PAGE), they were not used for 2D-PAGE. The pI value for each 2D-PAGE was measured and thus estimated manually (on the basis of the position in millimeters from the acidic edge of the IEF gel). For estimating the pH values, it was assumed that the edges of the short

range gels were 4.2 and 4.9 and of the broad range gel between 3.1 and 9.8. Always a sample of a male laboratory mouse (strain C57/BL6) was used as a reference.

IV.4.2.6 MALDI-TOF/TOF-MS/MS (*matrix-assisted-laser-desorption-ionisation – time-of-flight – tandem – mass-spectrometry*)

For mass spectrometric analysis, proteins were isolated from urine samples (pooled from each of the both male *Fukomys* individuals). The isolated proteins were concentrated by acetone precipitation. The amount of 200 μg of protein from each sample was resuspended in 200 μl IEF rehydration buffer and applied on IPG strip (11 cm, pH 3-10; BioRad Laboratories, Hercules, CA, USA). Second dimension was carried out as described above on pre-cast gels (BioRad Laboratories, Hercules, CA, USA) stained with Coomassie® G-250 (SimpleBlue™ Safe Stain, Invitrogen Life Technologies, Paisles, UK). The major spot with the molecular weight around 20 kDa was excised from the gel and subjected to trypsin digestion. The gel spot was washed twice with 100 μl of 50 mM ammonium bicarbonate buffer in 50% acetonitrile and let to dry. Five nanogram of trypsin (Promega) in 10 μl of 50 mM ammonium bicarbonate and 10 μl of 97% ^{18}O water were then added and the samples were incubated overnight at 37° C. Tryptic peptides were extracted with 1% trifluoric acid in 30% acetonitrile. The drop of the extract was placed on the MALDI target plate and allowed to dry. Then, it was covered with 20 mg/ml of α-cyano-hydroxycinnamic acid solution in 80% acetonitrile.

Tryptic peptides for *de novo* analysis were separated on PepMap 100 C18 RP column (3 μm particle size, 15 cm long, 75 μm internal diameter; Dionex) with a gradient of 5% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid to 80% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid using Ultimate 3000 HPLC system coupled to Probot microfraction collector (Dionex). Matrix solution (20 mg/ml α-cyano-hydroxycinnamic acid in 80% acetonitrile) was added in the ratio 1:3.

MS spectra were obtained using 4800 Plus MALDI TOF/TOF analyser (Applied Biosystems/MDS Sciex) equipped with Nd: YAG laser (355 nm, firing rate 200 Hz). MS/MS was performed with 1 kV collision energy and operating pressure of collision cell 10^{-6} Torr. Spectra were interpreted manually using double peaks formed by $^{16}O/^{18}O$ to distinguish y-ion series. The series of b-ions was matched to corresponding y-ion series by subtraction of y-ion mass from parent ion mass.

IV.4.2.7 Statistical test

Kruskal Wallis ANOVA for independent samples was applied to compare mean total urinary protein concentrations (Bradford test) and to asses differences between the tested animals of different genera, because the data were not normally distributed (Kolmogorov-Smirnov test). The Mann-Whitney *U*-test for independent samples was applied to compare mean total urinary protein concentrations (Bradford test) between males and females. All statistical analyses were performed with SPSS (version 14). Means are given as $\bar{x} \pm$ SD.

IV.4.3 RESULTS

IV.4.3.1 Bradford test

The mean total urinary protein concentrations of the individuals of all the tested subterranean species were 0.45 ± 1.09 SD mg/mg creatinine (Cr) (cf., appendix tab. VII.D1) with no significant differences between males and females (tested separately for each genus): Mann-Whitney *U*-test (*Fukomys* p = 0.90, *Spalax* p = 0.23, *Spalacopus* p = 0.37; cf., appendix tab. VII.D2) and with comparable values at the genus level (Kruskal Wallis one-way ANOVA, $\chi^2 = 3.168$, p = 0.205) (fig. IV.4.2). The urinary protein concentrations of the C57BL/6 laboratory mouse (12.4 ± 4.4 SD mg/mg Cr) were significantly higher than the concentrations of the tested subterranean species (Kruskal Wallis one-way ANOVA, $\chi^2 = 49.62$, $p < 0.001$) (fig. IV.4.2). Differences in urinary protein concentrations between male and female C57BL/6 mice were significant (Mann-Whitney *U*-test, Z=-3.10, p = 0.001). In contrast to the generally low values found for the subterranean rodents, there were three queens of *Fukomys* mole-rats with high total protein concentrations (individual #3: 2.7 mg/mg Cr, individual #8: 2.4 mg/mg Cr, and individual #17: 7.3 mg/mg Cr). The higher concentrations in these samples were also apparent in the SDS-PAGE (fig. IV.4.3).

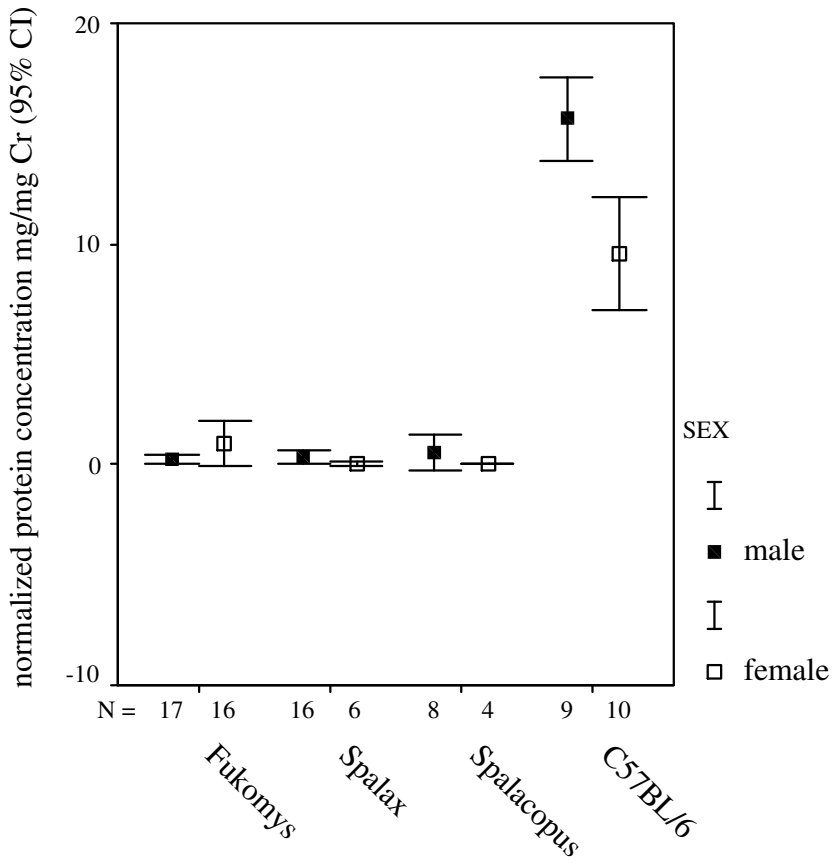


Fig. IV.4.2: Differences between genera (and sexes) in normalized total urinary protein concentrations (mg/mg Cr) between *Fukomys* spp., *Spalax ehrenbergi* species complex, *Spalacopus cyanus,* and laboratory mouse strain (C57BL/6). Filled squares represent differences in means between males, unfilled squares represent differences in means between females of different species with error bars (with 95% lower and upper confidence interval, CI; $\alpha = 0.05$). Cr = creatinine.

IV.4.3.2 SDS-PAGE

In the qualitative SDS-PAGE analysis of urine samples of the three subterranean genera under study, only *Fukomys* showed one or two consistent bands (approximately ~22.5 kDa) (fig. IV.4.3). In *Spalax,* only the samples of two males (out of 25 animals) had one band of approximately ~19-20 kDa (fig. IV.4.4). None of the *Spalacopus* specimens revealed a band in the MUPs range or lipocalin mass (cf., tab. IV.4.1).

Given that all the *Fukomys* individuals showed a band in the MUPs mass range, their SDS-PAGE pattern were analysed in order to investigate polymorphism related to species, sex, or reproductive status (e.g. queens versus non-reproductive daughters). No protein mass polymorphism could be detected for *Fukomys* with this method. Band patterns in the range of 22-23 kDa consisted mainly of two bands (23 out of 31 samples); all other animals showed a pattern of one band (8 out of 31) (fig. IV.4.3).

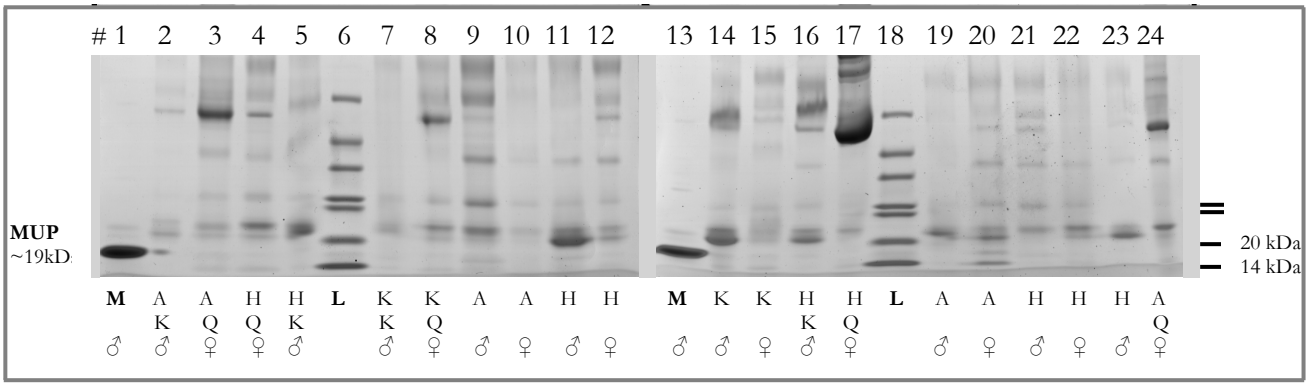


Fig. IV.4.3: SDS-PAGE (precast 4-15%) from males (♂), females (♀), kings (K) and queens (Q) of *F. anselli* (A), *F. kafuensis* (K) and their hybrids (H). Migration of urinary proteins from top to bottom. # = animal number, Sigma-7-ladder (L) and laboratory mouse, C57BL/6 (M) as internal (qualitative) standards. Males that were not kings were non-reproductive males (nrm). Females that were not queens were non-reproductive females (nrf).

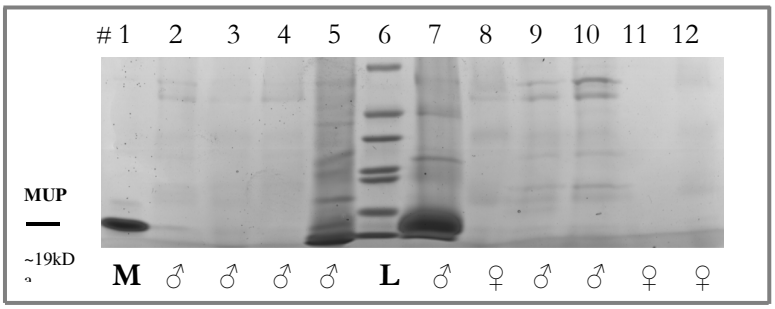


Fig. IV.4.4: SDS-PAGE of males and females of *Spalax ehrenbergi* species complex. # = animal number, Sigma-7-ladder (L) and laboratory mouse, C57BL/6 (M) as internal (qualitative) standard.

IV.4.3.3 2D-PAGE

The short range 2D-PAGE (isoelectric point pI = 3.9-5.1) revealed some similarity only between dot 1 of the laboratory mouse (C57BL/6) and dots 5 and 6 of *Fukomys* (#11 ♂). They differed slightly in mass, by about 3 kDa and the pI was also different (fig. IV.4.5). None of the dots could be detected in the three other *Fukomys* specimens (e.g. #14 ♂, fig. IV.4.3).

In the broad range 2D-PAGE (pI = 3-10), two dots (dot 1 and 2) of the tested *Fukomys* specimen (#11 ♂) were nearly congruent to dots 1 and 2 of the laboratory mouse strain, although they differed slightly in both parameters: in mass by approximately 1-3 kDa and in the pI (cf., fig. IV.4.3, appendix tab. VII.D3). The remaining two dots of the laboratory mouse could not be correlated with the dots found in *Fukomys* urine samples.

Of the two tested *Spalax* individuals, dot 3 of animal #7 ♂ had the same range in both parameters as the *Fukomys* and *Mus* specimens (fig. IV.4.6). None of the other numerous dots of this individual and any of the dots of individual #5 exhibited similar characteristics to the dots of the laboratory mouse or those of *Fukomys*.

All the tested *Fukomys* mole-rats revealed a similar pattern: dot 1 was roughly similar to the laboratory mouse, anyhow slightly different in mass (20-21 kDa) and pI, dot 2 was present in two male *Fukomys* individuals (#11 and #14), dot 3 in male #11, #14 and one queen (#4), and the characteristics of dot 4 in males #11 and #14 were similar to those in the laboratory mouse (appendix tab. VII.D3). In summary, those three dots might be typical for *Fukomys* mole-rats. The two dots that more or less fit the patterns of laboratory mouse strain could be detected just in one out of four *Fukomys* specimens.

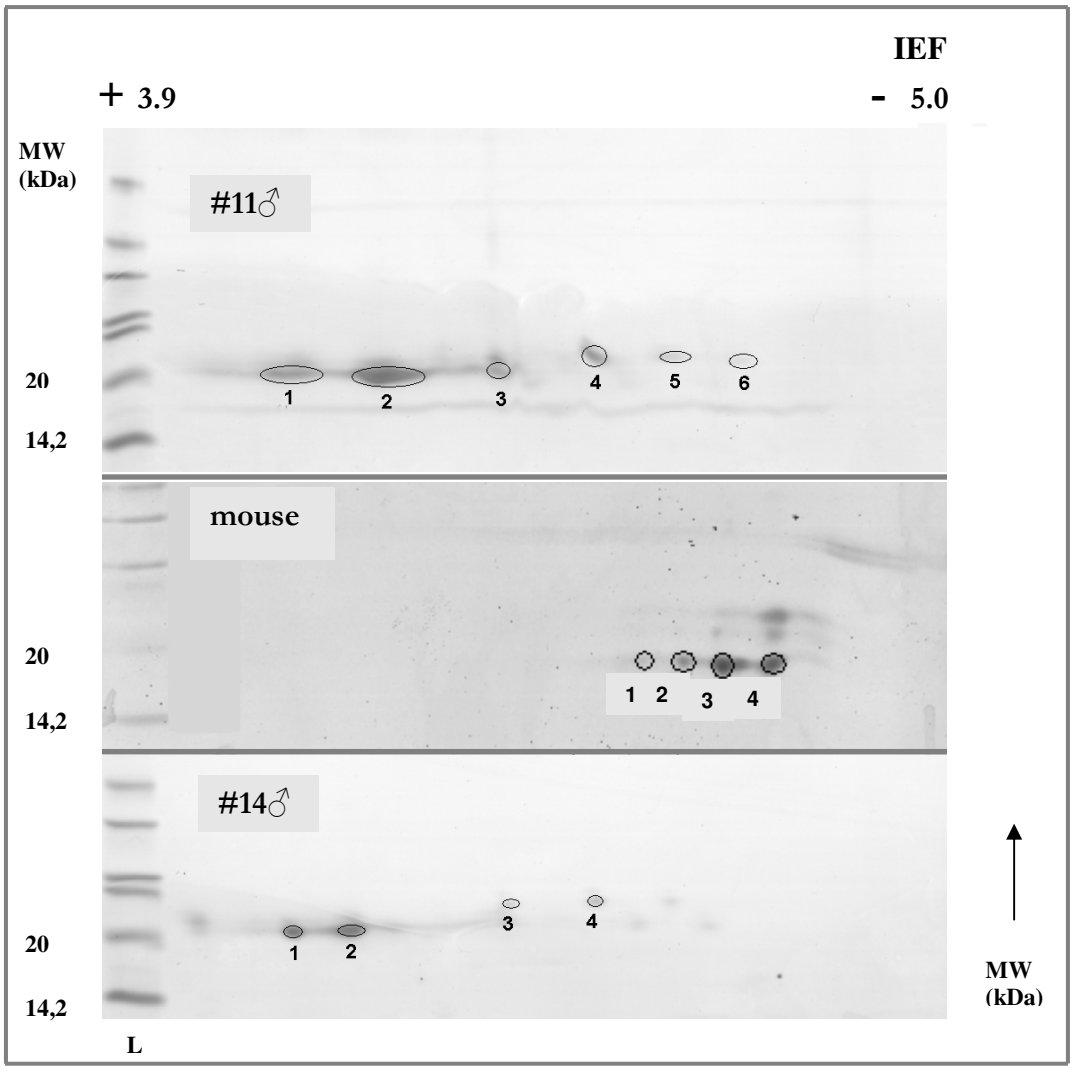


Fig. IV.4.5: 2D-PAGE of urine from two male *Fukomys* individuals and the house laboratory mouse C57BL/6. Isoelectric focussing (IEF) was conducted with low range stripes with the isoelectic point (pI) ranging between 3.9-5.1. Sigma-7-ladder (L). Molecular weight (MW) in kiloDalton (kDa). Animal numbers, #14 and #11, refer to numbers of the animals in fig. IV.4.3 (above the migration trace of the SDS-PAGE). + indicates the anode and - indicates the cathode of the IEF cell.

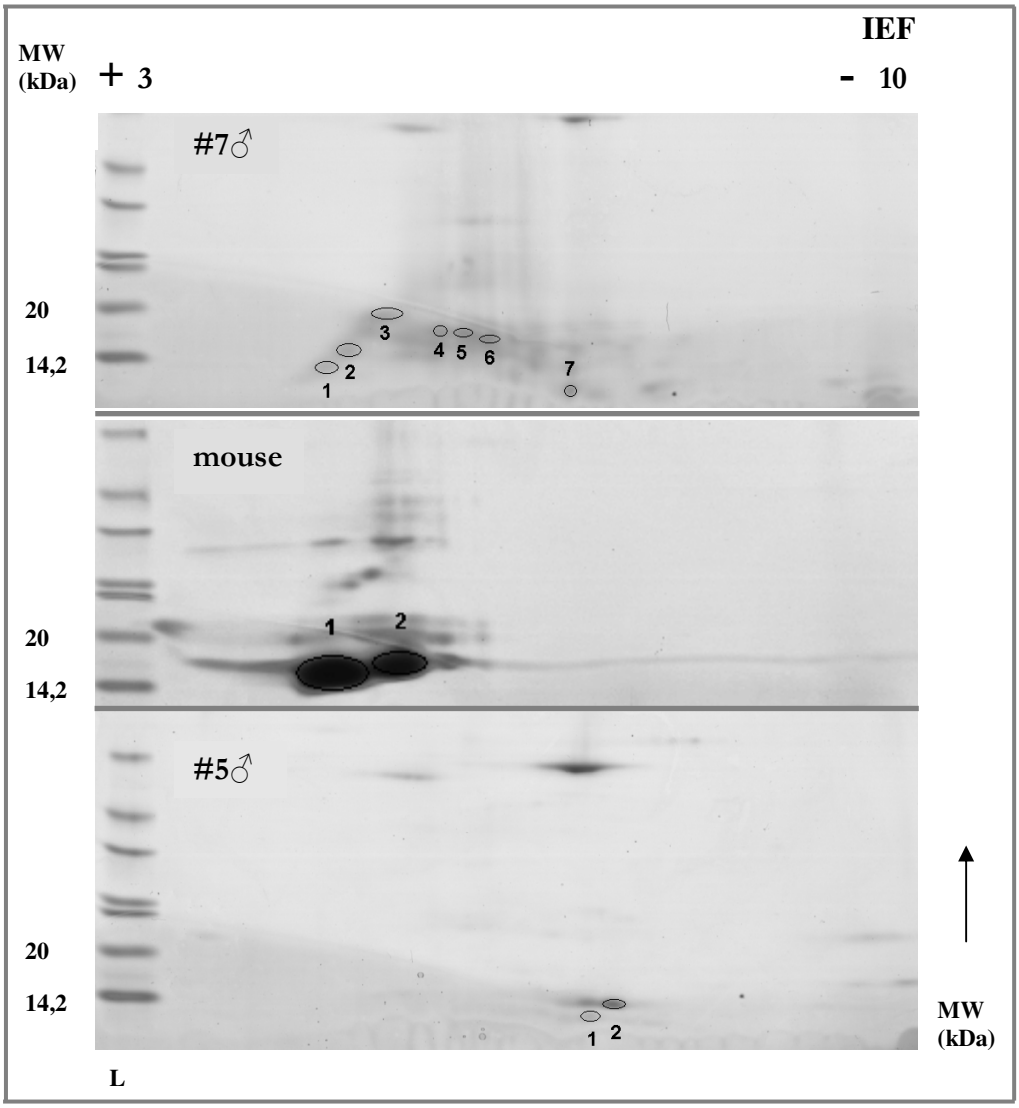


Fig. IV.4.6: 2D-PAGE of urine from two male *Spalax carmeli* and the laboratory mouse C57BL/6. Isoelectric focussing (IEF) was conducted with broad range stripes with the isoelectic point (pI) ranging between 3-10. Sigma-7-ladder (L). Molecular weight (MW) in kiloDalton (kDa). Animal numbers, #5 and #7, refer to numbers of the animals in fig. IV.4.3 (above the migration trace of the SDS-PAGE). + indicates the anode and - indicates the cathode of the IEF cell.

IV.4.3.4 MALDI-TOF/TOF-MS/MS (*matrix-assisted-laser-desorption-ionisation – time-of-flight – tandem – mass-spectrometry*)

Peptide mass fingerprints were matched against a sequence database to identify the proteins present in the 20 kDa spot (dot 2) of the 2D-PAGE gel of *Fukomys* (appendix tab. VII.D3, fig. IV.4.5). Note, for concentrating the protein of dot 2, it was necessary to pool the most appropriate two specimens (of male #11 and 14 of *Fukomys)* before tryptic digestion. No significant hits were obtained based on peptide masses. Subsequent *de novo* sequencing revealed the sequence of the peptide, which showed substantial similarity to the sequence of hamster aphrodisin (GI:1168469, BLAST, basic local alignment search tool, www.ncbi.nlm.nih.gov/blast). The fragment differs from the corresponding aphrodisin sequence by four amino acid residues. Changes are indicated in grey: YLAADNVEKIEEGGELR (fig. IV.4.9).

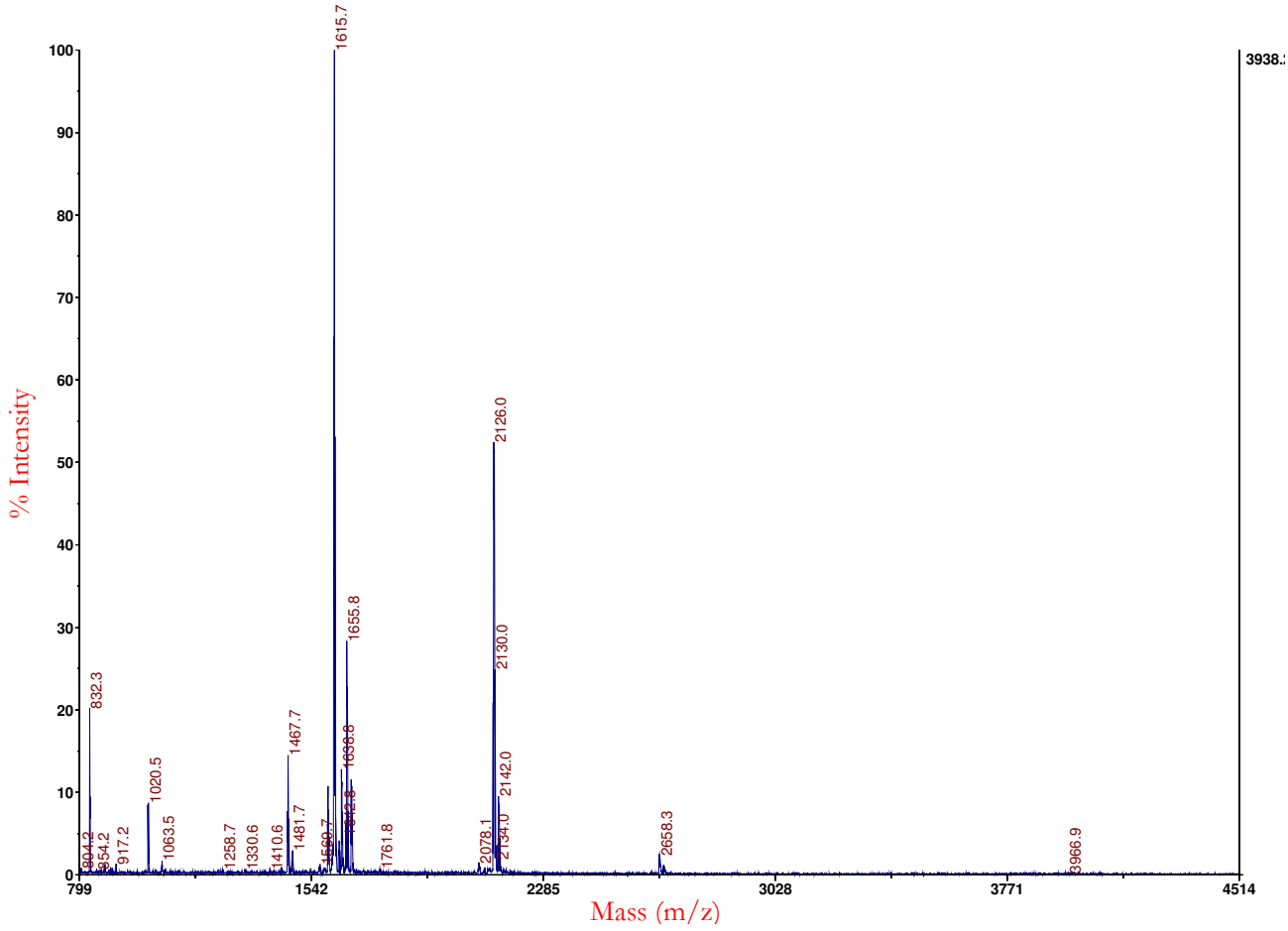


Fig. IV.4.7: MALDI-TOF mass spectrum of the peptide mass fingerprint (pmf) after tryptic digest. The peak with a mass of 2,126 Da was the peptide fragment delivering ion-series, which could be matched with high similarity to the hamster aphrodisin by the alignment tool (BLAST) of NCBI.

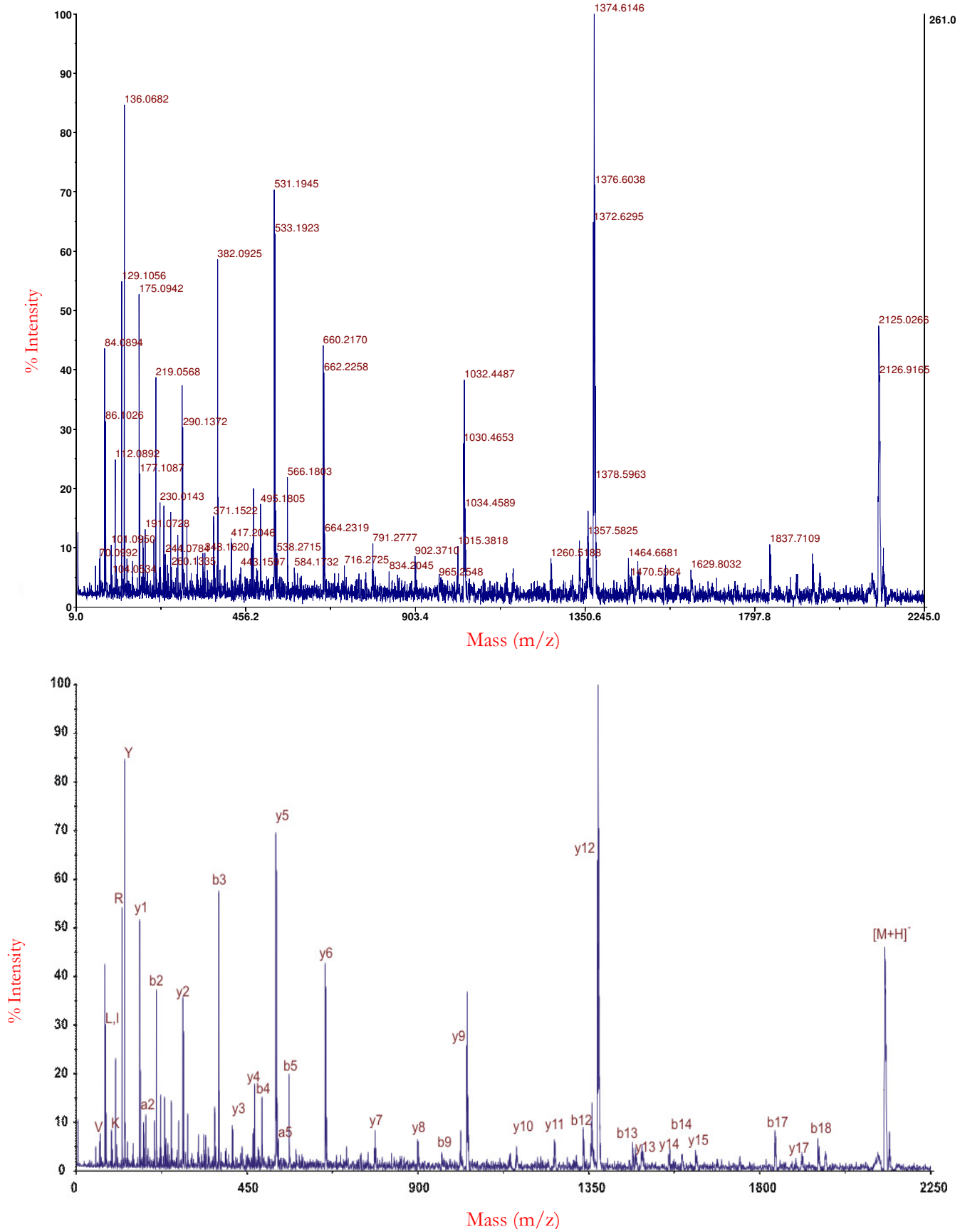


Fig. IV.4.8: Above MALDI-TOF mass spectrum of peptide fragments, obtained by tandem MS of the precursor peptide $[M+H]^+$-ion with a mass of 2,126 Da. Below the results of the fragmentation analysis are given as numbers of b- and y-fragments. Each peak indicates an ion of N- or C-terminal fragments of the percursor peptide (b and y-ion series). Manual calculation of each mass of the b- and y-fragments resulted in the amino-acid sequence, listed above (YLAADNVEKIEEGGELR).

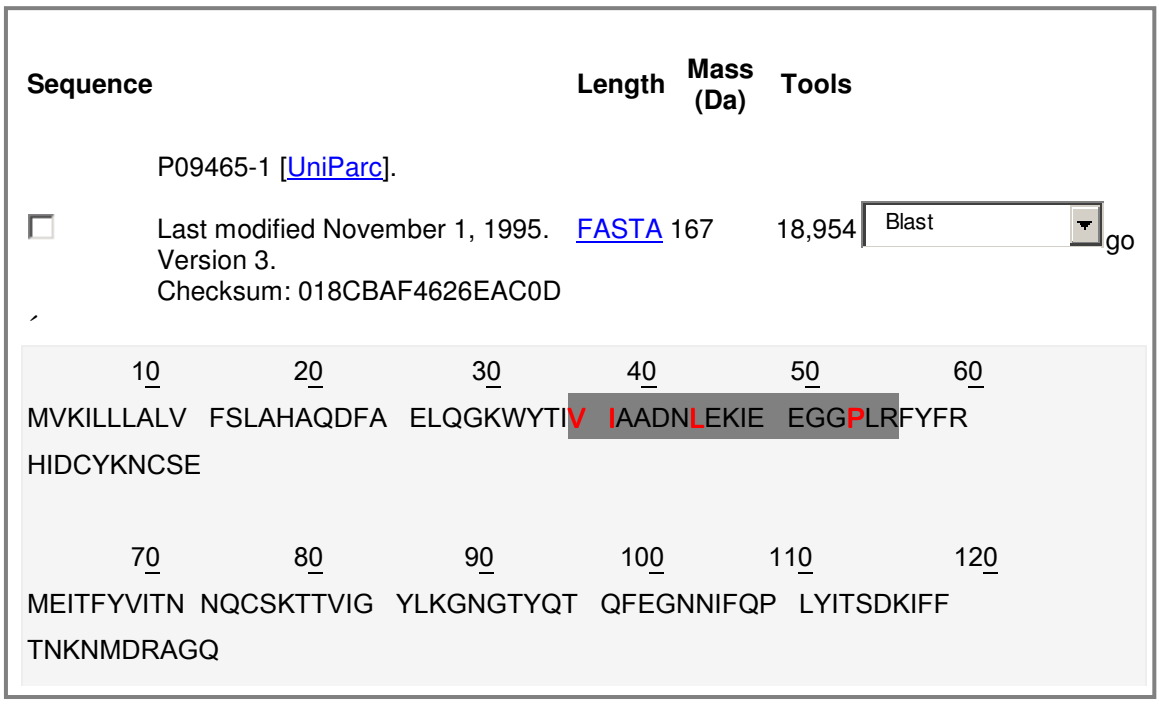


Fig. IV.4.9: Amino acid sequence information of hamster aphrodisine, obtained by BLAST (www.uniprot.org/uniprot/P09465) based on Henzel et al. 1988, Mägert et al. 1995, Vincent et al. 2001). The grey box of the amino acid sequence indicates the sequence aligned to the obtained data of MALDI-TOF-MS *de novo* sequencing. Changes to the amino-acids of aphrodisin are accentuated with red bold capitals.

IV.4.4 DISCUSSION

Basic findings

The biochemical analyses indicated the absence of MUPs in urine of the three tested rodent genera so far, but a lipocaline-like protein in *Fukomys* (tab. IV.4.1). No urinary protein in the mass range of ~16-21 kDa could be detected in *S. cyanus*. Of the 25 blind mole-rats tested, SDS-PAGE showed one band in the range of ~14 kDa for one individual blind mole-rat (#5, fig. IV.4.4) and one band in the ~18 kDa range for another individual blind mole-rat (#7, fig. IV.4.4). These bands could be expanded through 2D-PAGE to reveal in the first individual two dots (~11 and ~14 kDa) and in the second individual several dots ranging from ~11 to ~20 kDa (cf., fig. IV.4.6). High urinary protein excretion can be an indication of proteinuria (e.g. Rustom et al. 1998). Although there were no overt signs of illness at the time the individuals' urine was collected, illness could not be excluded, particularly since all other 23 *Spalax* samples showed no protein bands. One or two bands in the mass-range of ~22-23 kDa could be identified in all the *Fukomys* samples by SDS-PAGE. There was, however, no indication of mass polymorphism with respect to sex, species, or reproductive status (fig. IV.4.3). The analyses of 2D-PAGE further demonstrated in two *Fukomys* males two dots with a roughly comparable pattern compared to the laboratory mouse (dot 1 and 2; fig. IV.4.5, appendix tab. VII.D3). The typical dot pattern in *Fukomys* consisted of three dots that were in the range of approximately 4.0-4.5 pI with a mass of ~20-23 kDa.

Thus far, a protein of ~20-21 kDa by 2D-PAGE could be characterized. This protein spot (dot 2 of 2D-PAGE) was excised and pooled from two males of *Fukomys* mole-rats for tryptic digestion, since the concentration of this spot from only one individual was too low for further analysis. Although no significant hits were obtained based on peptide masses by the database match (BLAST of NCBI), a mass spectrum of an aphrodisin typical peptide with a mass of 2,126 Da (fig. IV.4.7) could be proved after tryptic digestion by the MALDI-TOF *de novo* analysis. The subsequent analysis of the 2,126 Da mass (precursor) peptide yielded ion-series, whose amino-acid sequence (YLAADNVEKIEEGGELR) produced significant alignment (BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi) with a significant similarity rather to the hamster aphrodisin (VIAADNLEKIEEGGPLR, fig. IV.4.8 above; Henzel et al. 1988, Mägert et al. 1995, BLAST www.uniprot.org/uniprot/P09465), than to a MUP (ILASDKREKIEDNGNFR, Henzel et al. 1988) or any other lipocaline sequence; the obtained sequence aligned to the query sequence of aphrodisin with the highest score (347 Bits) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Altogether, a sequence of

17 amino-acids with only 4 substitutions compared to the amino-acid sequence of aphrodisin (fig. IV.4.9) was obtained, whilst only 8 of the estimated peptide sequence matched to the MUP-peptide sequence (Henzel et al. 1988).

Since the sequences of even short runs of amino-acids can be used to search databases using alignment tools such as BLAST (Beynon et al. 2007) it is justified to conclude on the base of the short sequence of 17 amino-acids (obtained by MALDI-TOF-MS *de novo* analysis) with high probability on aphrodisin in *Fukomys* mole-rats. In fact, until now the obtained results demonstrate that none of the subterranean rodent genera under investigation produced MUPs in their urine, but *Fukomys* produced aphrodisin at low concentrations. It should however, kept in mind that the excision procedure left certain protein rests of the 20 kDa spot (dot 2) of the 2D-PAGE into the gel. Moreover, dot 1, 3, and 4 were not analysed yet by MALDI-TOF-MS *de novo* analysis, not allowing us to exclude the excistence of MUPs in *Fucomys* completelely.

Protein concentrations and reproductive status

Although the urinary proteins of *Fukomys* were identified as aphrodisin, the Bradford analysis revealed very low mean total urinary protein concentrations: They were 39 times lower in *Fukomys* than in the C57/BL6 laboratory mouse and even 46 times lower, when all the analysed urine samples of the subterranean species were considered (cf., appendix tab. VII.D2). Given that 99% of the protein excretion in *Mus* are composed of MUPs (Humphries et al. 1999), this proportional imbalance theoretically even increases, since in all the subterranean individuals, the total urinary protein content is composed of several proteins (visible in several protein bands of SDS-PAGE; cf., fig. IV.4.3). Despite the fact, that the tested subterranean rodents do not drink free water, they produce large amounts of urine, a fact that might explain the low protein concentrations. Because urinary proteins of male laboratory mice were much more concentrated than those of females, the sex-dependent differences in the laboratory mouse were larger than across the tested subterranean species (fig. IV.4.2). Both types of data (Bradford and SDS-PAGE) thus demonstrate the substantial differences in the amount of urinary proteins in laboratory mice in comparison to the tested subterranean species

To date, aphrodisin is known from the vaginal discharge of the female golden hamster *Mesocricetus auratus* (e.g. Henzel et al. 1988, Mägert et al. 1999, Briand et al. 2004). It elicits mounting behaviour in the males, mediated by direct physical contact (Agosta 1992). A recent study of Beynon et al. (2007) identified aphrodisin also in urine of the Roborovski hamster, *Phodopus roborovskii*. The fact, that it was found also in equal amounts in urine of males and females should be particularly interesting here. Intriguingly and in concordance with our results, aphrodisin was detected also in the urine of a rodent, taxonomically less

related to the hamsters, the bank vole (*Myodes glareolus*) (Beynon et al. 2007), confirming that aphrodisin seems to be more widespread in rodents than previoiusly thought. More importantly, it is not limited to female vaginal discharge. Rather, it is expressed in larger amounts in male bank voles, indicating a strong sexual dimorphism, which, however, cannot be confirmed for *Fukomys*, because of the limited sample size of the proteomic investigations (peptide mass fingerprint). The data were obtained from a single specimen, composed of the concentrated 2D-PAGE-dot of two male individuals. Anyhow, also by SDS-PAGE, any sexual mass polymorphism could be found. Moreover, contrary to *Fukomys* mole-rats, Beynon et al. (2007) discovered that the proteins of the other tested rodents had one trait in common: Urinary proteins are expressed in substantial concentrations.

Thus, if aphrodisin would take on the role of MUPs in carrying information about, e.g. sex or the reproductive or social status, it should be reflected either in the amount of urinary proteins (which was not the case) or in mass and/or charge polymorphism. No such polymorphism, i.e. no consistently different protein band pattern in the SDS-PAGE was detected (cf., fig. IV.4.3), yet, to date, no band patterns were analysed by IEF.

Function of aphrodisin in Fukomys (ligand-carrier-sytem?)

Although any consistent differences in urinary proteins between animals of different gender or status could not be detected, there are large differences in concentrations of steroids between female *Fukomys* mole-rats of different reproductive status: The queens exhibited significantly higher estradiol and progesterone concentrations in urine than their sexually quiescent daughters. Moreover, a potential lipophilic ligand 4-nonanone was identified as a queen-specific compound by chemo-analytical HS-SPME-GC-MS-investigations (cf., chapter IV.3.1.3.4; tab. IV.3.4). Both such conspicuous dimorphisms are however, not reflected in the amount of aphrodisin, thus disqualifying most probably aphrodisin to be a candidate for a carrier of both compounds. Intriguingly, by chemo-analytical HS-SPME-GC-MS-analysis a further probable ligand, 1-hexadecanol, could be identified in urine of non-reproductive female *Fukomys* mole-rats (cf., chapter IV.3.1.4). This organic compound was proved to be a ligand of aphrodisin in the golden hamster; although the role of this lipophilic ligand remains speculative, the transport through the aqueous mucus to the vomeronasal receptor cells is favored (Briand et al. 2004). Moreover, Zhang et al. (2007) identified this compound to be a male-specific ligand of MUP, being secreted by the preputial glands in the house mouse. Contrary, in *Fukomys* this compound could be detected in the urine, solely in reproductively quiescent females (discussed partly in chapter IV.3.1.4; cf., tab. IV.3.4). Advertising to be reproductively quiescent or having no reproductive experience by chemical signals, seems not to be

advantageous, e.g. when encountering a potential male mating partner. On the other hand, within the family, signalling to be a sexually quiescent daughter by 1-hexadecanol, additionally and essentially to individual discrimination, would be reasonable for brothers and the father in the context of inbreeding avoidance. Also, one could speculate that this signaling could prevent harassment by the mother, the queen. Therefore in the latter context, it seems comprehensible, that this compound has a chemosignalling capability in *Fukomys*. But since the presence of 1-hexadecanol to date does not reflect any dimorphism of aphrodisin, i.e. different band pattern in SDS-PAGE between both female categories, its role as a ligand of aphrodisin in *Fukomys* mole-rats still remains to be investigated in order to detect e.g. isoforms. This holds true also for ß-caryophyllene, found in *Fukomys* also as a sexually dimorphic trait (this study): Males excrete it into urine in significantly higher concentrations than females (regardless of the reproductive status, i.e. king or non-reproductive males). Up to now, on proteomic level I again could, however, not identify any sexual dimorphism in the band pattern of aphrodisin. Perhaps both compounds don't need any carrier system.

To refine the picture that we can deduce from *Fukomys* mole-rats with respect to the ligand-carrier-system of semiochemicals: Aphrodisin was identified in low amounts in the urine, without any sexual or reproductive dimorphism. Furthermore, several organic compounds were detected, with sexual dimorphism and/or with reproductive dimorphism. Implying olfactory importance of those identified organic compounds for *Fukomys* mole-rats, in urine they perhaps operate without a carrier system. This might be explained as a consequence of their subterranean lifestyle, since in the subterranean environment, physical constraints differ conspicuously from the aboveground conditions. Two important factors found underground are the low gas ventilation and high humidity (Burda et al. 2007). After urine is excreted, semiochemicals should evaporate relatively slowly in such a windless environment. Hence in the underground ecotope urinary lipocalines, such as aphrodisin, are probably useless, or only low concentrations are needed to decrease the volatility of semiochemicals and thus to prolong their odourous effects. On the other hand, the subterranean ecotope is not the exclusive explanation for abondoning or not evolving the lipocaline carrier-protecting system, since the wood mouse (*Apodemus sylvaticus*), an aboveground living rodent, lacks urinary proteins in the 18-20 kDa range typical of lipocalins (Stopka, unpublished data). To get deeper insight into a probable carrier-task of aphrodisin, further for isoforms by MALDI-TOF sequencing need to be searched. Furthermore, it need to be understood, whether aphrodisin in *Fukomys* is expressed also in glands, such as in the Barholin's gland, parotid gland, vagina, and uterus as demonstrated in the golden hamster (Mägert et al. 1999).

Olfactory bioassays using ano-genital secretions were always stronger, than the outcome obtained by urine (cf., chapter IV.1). Therefore, a transport mechanism for aphrodisin (e.g. in body secretions) in reproductive contexts cannot be excluded. Among lipocalins, aphrodisin is highly homologue (40% sequence identity) with OBP (odourant binding proteins) of rats (Briand et al. 2000b). OBP is thought to carry volatile odourants towards olfactory receptors through the nasal mucus. In *Fukomys,* it is imaginable (especially in low distance communication) that aphrodisin transfers one/or a blend of such small lipophilic odourous molecules from the sender mole-rat directly to the OBP of the receiver mole-rat by direct (and personally often observed) body contact. Thus aphrodisin could help to overcome the aqueous barrier for lipophilic odourous molecules or even act as a pheromone on its own. Surely, these considerations remain speculative and further research is needed.

Individual discrimination

Apart from sexual olfactory communication, all the subterranean rodent species studied here, are known to discriminate between individuals on the basis of odours in their ano-genital and/or ventral secretions that may also include some urine (in *Spalax*, Todrank & Heth 1996, Heth & Todrank 2000; in *Fukomys* ano-genital odour, Heth et al. 2002 and 2004; and in *Spalacopus cyanus*, Hagemeyer & Begall 2006). Blind mole-rats of the genus *Spalax* moreover, are known to differentiate between individuals by urinary odours. Besides information about individuality, urine from *Spalax* also provides information about the population and species (Heth et al. 1996a, Todrank & Heth 1996, Heth & Todrank 2000), thus eliminating lipocalins, like MUPs or aphrodisin, as exclusive candidates for transmission of these facets of identity.

Recent studies also acknowledged that MUPs could not convey individual identity in species where there was no polymorphism in the MUPs, as in *Mus macedonicus* (Robertson et al. 2007), and where the patterns of urinary lipocalins across individuals were "remarkably consistent", as in *M. spretus* as well as in wild Norway rats, *Rattus norvegicus* (Beynon et al. 2007). Referring to *Fukomys*, recent results of chemo-analytical HS-SPME-GC-MS and behavioural investigations (chapter IV.3) in parts are consistent with those findings: Firstly, to date no individual polymorphism of aphrodisin was detected in *Fukomys* mole-rats. All individuals showed the same band pattern of urinary aphrodisin in the SDS-PAGE, which still have to be verified by ongoing proteomic investigations (as described above). Thus, if aphrodisin plays a role in chemosignalling, conveying individual identity, it seems not to be involved in this context. Secondly, urinary volatiles do not represent individual differences, evidenced by relatively similar peak pattern of the gas-chromatogram between different individuals (even between families).

Thirdly, contrary to the above mentioned muroid species, on the behavioural level urine seems not to be very informative for individual identification in *Fukomys* (cf., chapter IV.1).

Method

The amino-acid sequence (17 amino-acids long), obtained by *de novo* sequencing from the precursor peptide after tryptic digest is very short and is far from providing information about the whole protein. The match of this sequence to aphrodisin is high, but since aphrodisin of the golden hamster has the amount of 151 amino-acids (Henzel. et al. 1988) many uncertainties according to 134 undiscovered sequences need to be investigated by sequencing more peptide precursors. Therefore many features, e.g. the amount of amino-acids substitutions and the resulting different three dimensional fold of the detected protein of *Fukomys* remain to be elucidated. This should be conducted with many individuals belonging to different sex and reproductive status in order to resolve isoforms. Moreover, the precise mass of the whole protein and ligand binding characteristics remain to be discovered.

In summary, in species lacking MUPs completely or with extremely low concentrations of urinary lipocalins, like the rodent species in the present study, there would have to be some other and/or additional mechanism for disseminating information about biological status (e.g. dominance or reproductive maturity), as well as individual identity. Although the behavioural research on *Fukomy*s and the species under study focused primarily on aspects of individual identity and responses to genetic differences, *Fukomys* and *Spalax*, both responded to differences in urine that are indicative, for example, of reproductive status (Heth et al. 1996b and chapter IV.1). Additional research is necessary to determine whether aphrodisin facilitates communication of information about reproductive status in *Fukomys*; it is, however, clear that this would not be possible in *Spalax* or *Spalacopus* – species that lack both MUPs and aphrodisin.

V CONCLUSIONS & OUTLOOK

This interdisciplinary dissertation thesis contributed to a broader understanding of the olfactory communication in *Fukomys* mole-rats with respect to information about sex and individual and family identity. Moreover, light was shed on olfactory communication processes, subjected to dynamic variances due to the reproductive status. Those intentions were applied by four different methodological approaches: Behaviourally, by habituation-generalization and preference tests with ano-genital and urine odour, supplemented by colpocytological and endocrinology methods, i.e. levels of circulating urinary steroids between different animals categories. Additionally, both, a chemo-analytical HS-SPME-GC-MS and a proetomic approach by MALDI-TOF/TOF-MS/MS on urine, highlighted in parts underlying metabolic processes in *Fukomys*, confirming the results of the behavioural assays.

In contrast to ano-genital odour, there is persuasive evidence (behavioural and for urine also chemo-analytical) that urine does not contain odourous information in the context of kin recognition, i.e. individual identity, and thus does not confirm the hypothesis of odour-genes covariance. Interindividual variations were found to depend on sex (male or female) and reproductive status (queen or non-reproductive female) and not on individuality per se. *Fukomys* individuals seem to be able to differentiate subtle odour differences even between unknown strangers. The prerequisite of such ability, however, is the possibility to learn the odourous template by former encounters, which was not the case. Similarly irritating and enigmatic is the finding that urine odour originating from known siblings could not be differentiated from the odour originating from a stranger.

Those heterogeneously appearing results challenge us to offer explanations: Firstly, the urine odour of close relatives contains information about high genetic similarity and/or pronounced differences (not supported by chemo-analytical results) dependent on the degree of kinship. Secondly, the self-reference and phenotype matching process cannot be applied for urine, i.e. subjects do not have the possibility to associate the urine odour with the individuals' odour, because urine is deposited in the common latrine and does not stick at the individuals' body in sufficient amounts. The third explanation questions the habituation-generalization test design for this source of odour in general. As discussed in chapter IV.1, multiple exposition to "monotonous" same-sex odours (delivering always the same information) may result in decreasing motivation levels, biasing the statistical

outcome. This, in fact, could be demonstrated by low sniffing times during the second test-trial, which was designed to rule out low motivation levels.

The chemo-analytical approach is thus in part consistent with the behavioural results: It could be demonstrated that the 51 compounds constituting the volatile urinary pattern (of total ion chromatograms) in non-reproductive females and non-reproductive males, respectively, were quite similar even between different families. Also the urine of families, revealing 41 different compounds, does not contribute to family discriminative odour. Such similar urinary pattern of course does not explain, why animals seem to be able to discriminate between subtle urine odour differences without learning process (phenotype matching) by former encounters.

It should be, however, kept in mind, that probably not all the existent compounds were extracted by the DVB/CAR™/PDMS™Stable Flex™-fibre. Substances with different polarity and/or volatility thus may remain chemically undetected, probably explaining the ability of detecting odour differences which to date appear to be subtle or even absent but which in reality for the mole-rats are more pronounced.

In fact, diet could influence the urinary volatile composition, leading to an increase of certain urinary compounds when kept on the carrot diet and a decrease of others when kept on the potato diet. Contrary to laboratory conditions, in the field this may result in more pronounced different family odours, according to their food preferences and their dependence of food resources, namely the regionally occurring plants. However, this seems to be a rather theoretical possibility. We have to assume that neighbouring families in a given area most probably have also access to the same food and hence are influenced in the same way. Mole-rats which live in distinct areas with distinct food resources do not have the chance to encounter each other. In any case, it remains questionable, whether such diet-dependent family urinary differences are smelled and bear any relevant meaning to *Fukomys* mole-rats. Unfortunately, none of those "carrot-connected" peaks could chemically be identified so far.

The dependence on sex and reproductive status for interindividual variations is strongly supported by steroidal analysis, demonstrating in females high correlation between mean urinary estradiol and progesterone concentrations on one side and sexual activity on the other side. This additionally enables methodically to differentiate queens from their non-reproductive daughters by both circulating steroids. Moreover, colpocytological investigations showed no cyclic changes due to a lack of ovarian cycle and of spontaneous ovulation. Even after successful matings, no typical estrus-like cells appeared. Thus, *Fukomys* mole-rats are most probably induced ovulators.

Contrary to Damaraland mole-rats, non-reproductive females of Zambian *Fukomys* mole-rats under study are sexually not suppressed by the absence of the queen, which excludes suppression via "queen-signalling" pheromones. Rather, females most likely are primed solely by the whole range of sexual activity, since the lack of copulation (consequently connected with successful reproduction) seems to lead to low estradiol and progesterone concentrations. This (possibly together with daughters' urinary 1-hexadecanol, see below) should be one aspect to prevent breeding in non-reproductive females ensuring the incest avoidance. The signalling relevance of such single urinary volatile should be enlightened by behavioural assays. More difficult, however, would be to rule out the contribution of a potential odourous or even pheromonal blend.

Obviously, the reproductive status of the queens not only acts on altered steroidal metabolism (probably smelled by males), it acts as well on further potential odourous urinary compounds, maybe enabling males to identify the female status by chemosensation. Besides four compounds metabolized in higher concentrations in dependence of the reproductive active status, 4-nonanone seems to be such a queen-specific urinary compound, whilst 1-hexadecanol was shown to be characteristic for the reproductively quiescent daughters. In respect to and in agreement with the bioassay (preference test), both compounds seem to contribute to different urinary blends, enabling e.g. strange males to prefer queens over sexually inexperienced females. Note that the behavioural outcome was stronger in tests with ano-genital odour than urine odour. Furthermore, in this way, daughters might signal (unintentionally) their reproductive state to fathers and brothers, beside their individuality and close relationship (most probably signalled by body excretions, not by urine) in order to maintain the incest avoidance. This is, however, rather speculative, because it was shown that forgetting per se was sufficient to release the incest avoidance (Burda 1995) and secondly, strange non-reproductive females (which also are characterized by this substance) are attractive for strange males. Another possibility would be that this odour suppresses aggressive harassment by the queen. Of course, the other possibility is that this is just a byproduct of the metabolism unaffected by female steroids (which would also explain why it is missing in males) and has no significant biological effect for communication

Both sexes differ in their urinary composition: Besides generally lower proportions of urinary volatiles compared to females, males display higher concentrations of ß-caryophyllene. The reproductive status of males is, however, not reflected by the urinary volatile profile. This missing difference is supported by similar testosterone concentrations in reproductive and non-reproductive males.

Until now, 51 different volatile urinary compounds of individuals could be detected by a new solvent free method of analytical sample extraction (SPME) of which five were identified by mass spectra whilst the others remain tentative or enigmatic so far. Compound classes, well known for their odourous potential in other species, constitute the volatile blend (maybe genus-typical) of *Fukomys* urine: terpenes, alcohols, aldehydes, ketones and esters.

A known sex pheromone of the golden hamster (*Mesocricetus auratus*), aphrodisin, was identified here also in the urine (contrary to vaginal discharge as in hamsters) of *Fukomys* mole-rats, but in atypically low concentrations. In contrast to the golden hamster, however, no mass polymorphism (concerning the bands of SDS-PAGE) with respect to individuality, sex, age or reproductive status could be revealed in mole-rats. Since aphrodisin does not exhibit dimorphic characteristics of the volatile urinary compounds, it probably does not play a ligand binding role (slowing down ligand release, prolonging and protecting mechanism of the volatiles). This is discussed on the background of specific characteristics of the subterranean environment. I suggest that ongoing investigations may reveal aphrodisin in higher concentrations in the vaginal discharge, which could explain at least in females its urinary diluted occurrence. The analysis of bladder urine would also help in this respect. On the other hand, until now, in mole-rats, it was extracted from only two males.

Those preliminary outcomes encourage deepening the proteomic insight, in order to address several open questions. For instance, the hitherto missing polymorphic traits of aphrodisin (given, that it corresponds to hitherto not analysed SDS-bands as well) should be proved by further IEF and MALDI-TOF-MS investigations, to narrowing the issue of the direct or indirect role of this lipocaline and its potential ligands (e.g. 1-hexadecanol and/or ß-caryophyllene). Genetic investigations (cDNA-sequencing) are needed to confirm and broaden the expressed amino-acids sequence, of which the current study identified a sequence of 17 out of 151 amino-acids so far. Additionally, beside searching aphrodisin also in females the source(s) of expressing tissues should be identified: For instance, the Barholin's gland, Harder's gland, parotid gland, vagina or uterus.

Finally, out of three different genera of subterranean rodents (*Spalax*, *Spalacopus, and Fukomys*), aphrodisin could be identified only in *Fukomys*, and its pheromonal relevance still has to be clarified. Obviously, in contrast to claims in many studies, signalling of individual identity in rodents is not constrained to lipocalines such as MUPs, since both *Spalax* and *Spalacopus* communicate kinship by means of urine.

This thesis provided a basis for narrowing down the molecular nature of olfactory communication in *Fukomys* mole-rats. Urine as a first step was checked on three different interdependent levels – steroidal, volatile and proteomic. Body secretions, for instance ano-genital odour or saliva remain, however, unstudied, although their analyses are expected to provide highly illuminating results, since the behavioural assays with ano-genital odour were always stronger. The new solvent free method of SBSE (stir bar sorptive extraction) should be the method of choice in this respect.

VI REFERENCES

ACHIRAMAN SH & ARCHUNAN G (2006) 1-jodo-2methylundecane, a putative estrus-specific urinary chemo-signal of female house mouse (*Mus musculus*). Theriogenology 66: 1913-1920.

AGOSTA WC (1992) Chemical communication. The language of pheromones. Scientific American Library, New York.

AKERSTROM B, FLOWER DR & SALIER JP (2000) Lipocalines: unity in diversity. Biochimica et Biophysica Acta-Protein Structure and Molecular Enzymology 1482: 1-8.

ALBONE EC (ed) (1984) Mammalian semiochemistry. The investigation of chemical signals between mammals. Wiley & Sons Press, Chichester, New-York, Brisbane, Toronto, Singapore.

ALLEN E (1922) The estrus cycle in the mouse. American Journal of Anatomy 30: 297-371.

ALTSCHUL SF & LIPMAN DJ (1990) Protein database searches for multiple alignments: Proceedings of the National Academy of Sciences USA 87: 5509-5513.

ANDREOLINI F, JEMIOLO B & NOVOTNY M (1987) Dynamics of excretion of urinary chemosignals in the house mouse (*Mus musculus*) during the natural estrus cycle. Experientia 43: 998-1002.

ARMSTRONG SD, ROBERTSON DHL, CHEETHAM SA, HURST JL & BEYNON RJ (2005) Structural and functional differences in isoforms of mouse major urinary proteins: a male-specific protein that preferentially binds a male pheromone. Biochemical Journal 391: 343-350.

AUGER J, ROUSSET S, THIBOUT E & JAILLAIS B (1998) Solid-phase microextraction gas chromatography direct deposition infrared spectrometry as a convenient method for the determination of volatile compounds from living organisms. Journal of Chromatography A 819: 45-50.

BALTUSSEN E, CRAMER CA & SANDRA PJF (2002) Sorptive sample preparation – a review. Analytical and Bioanalytical Chemistry 373: 3-22.

BAPPERT M & BURDA H (2005) Forgotten siblings or how the long memory of the eusocial giant mole-rat *Crytomys mechowi* (Rodentia: Bathyergidae) serves to maintain incest avoidance. Mammalian Biology 70s: p. 5.

BEGALL S (1999) Verhaltensökologische und genetische Analysen der Sozial- und Populationsstruktur von Coruros (*Spalacopus cyanus*, Octodontidae, Rodentia) aus Chile. PhD thesis, University of Essen, Essen, Germany.

BENNETT KL, LALLEY PA, BARTH RK & HASTIE ND (1982) Mapping the structural genes coding for the major urinary proteins in the mouse: combined use of recombinant inbred strains and somatic cell hybrids. Proceedings of the National Academy of Sciences USA 79: 1220-1224.

BENNETT NC (1994) Reproductive suppression in social *Cryptomys damarensis* colonies: A lifetime of socially-induced sterility in males and females (Rodentia, Bathyergidae). Journal of Zoology (London) 234: 25-39.

BENNETT NC, GUTJAHR GH & FAULKES CG (2007) The reproductive physiology and endocrinology of the African mole-rats: with special reference to southern African mole-rat species. In: BEGALL S, BURDA H & SCHLEICH C (eds) Subterranean rodents – News from underground. Springer Verlag, Berlin, Heidelberg, pp 61-78.

BERGER GF & SZOKA P (1981) Biosynthesis of the major urinary proteins in mouse liver: A biochemical study. Biochemical Genetics 19: 1261-1273.

BEYNON RJ, VEGGERBY C, PAYNE C, ROBERTSON DHL, GASKELL SJ, HUMPHRIES RE & HURST JL (2002) Polymorphism in major urinary proteins: Molecular heterogeneity in a wild mouse population. Journal of Chemical Ecology 28: 1429-1441.

BEYNON RJ & HURST JL (2003) Multiple roles of major urinary proteins in the house mouse, *Mus domesticus*. Biochemical Society Transactions 31: 142-146.

BEYNON RJ & HURST JL (2004) Urinary proteins and the modulation of chemical scents in mice and rats. Peptides 25: 1553-1563.

BEYNON RJ, HURST JL, TURTON MJ, ROBERTSON DHL, ARMSTRONG SD, CHEETHAM SA, SIMPSON D, MACNICOLL A, & HUMPHRIES RE (2007) Urinary lipocalins in Rodentia: is there a generic model? In: HURST JL, BEYNON RJ, ROBERTS SC & WYATT T (eds) Chemical Signals in Vertebrates vol. 11. Springer Verlag, New York, pp. 37-49.

BINGEL AS & SCHWARTZ NB (1969) Timing of LH release and ovulation in cyclic mouse. Journal of Reproduction and Fertility 19: 223 -229.

BOCSKEI Z, FINDLAY JBC, NORTH ACT, PHILLIPS SEV, SOMERS WS, WRIGHT CE, LIONETTI C, TIRINDELLI R & CAVAGGIONI A (1991) Crystallization and preliminary-X-ray data for the mouse major urinary protein and rat-alpha-2U globulin. Journal of Molecular Biology 218: 699-701.

BRENNAN PA & KEVERNE EB (2004) Something in the air? New insights into mammalian pheromones. Current Biology 14: 81-89.

BRIAND L, HUET JC, PEREZ V, LENOIR G, NESPOULOUS C, BOUCHER Y, TOTIER D & PERNOLLET JC (2000a) Odorant and pheromon binding by aphrodisine, a hamster aphrodisiac protein. Federation of European Biochemical Societies Letters 476: 179-185.

BRIAND L, NESPOULOUS C, PEREZ V, REMY JJ, HUET JC & PERNOLLET JC (2000b) Ligand-binding properties and structural characterization of a novel rat odorant-binding protein variant. European Journal of Biochemistry 267: 3079-3089.

BRIAND L, BLON F, TOTIER D & PERNOLLET JC (2004) Natural ligands of hamster aphrodisin. Chemical Senses 29: 425-430.

BROWN RE, SINGH PB & ROSER B (1987) The major histocompatibility complex and the chemosensory recognition of individuality in rats. Physiology & Behavior 40: 65-73.

BROWN AE, RIDDICK EW, ALDRICH JR & HOLMES WE (2006) Identification of (-)-ß-caryophyllene as a gender-specific terpene produced by the multicolored asian lady beetle. Journal of Chemical Ecology 32: 2489-2499.

BRUCE HM & PARROTT DM (1960) Role of olfactory sense in pregnancy block by strange males. Science 131: 1526.

BURDA H (1989) Reproductive-Biology (behavior, breeding, and postnatal-development) in subterranean mole-rats, *Cryptomys hottentotus (*Bathyergidae). Zeitschrift für Säugetierkunde 54: 360-376.

BURDA H (1990) Constraints of pregnancy and evolution of sociality in mole-rats. With special reference to reproductive and social patterns in *Cryptomys hottentotus* (Bathyergidae, Rodentia). Journal of Zoological Systematics and Evolutionary Research 28: 26-39.

BURDA H (1995) Individual recognition and incest avoidance in eusocial common mole-rats rather than reproductive suppression by parents. Experientia 51: 4411-4413.

BURDA H (1999) Syndrome of eusociality in African subterranean mole-rats (Bathyergidae, Rodentia), its diagnosis and aetiology. In: WASSER SP (ed) Evolutionary theory and processes: Modern perspectives. Kluwer Academic Publication, NL-Dordrecht, pp 385-418.

BURDA H, HONEYCUTT RL, BEGALL S, GRÜTJEN O & SCHARFF A (2000) Are naked and common mole-rats eusocial and if so, why? Behavioral Ecology Sociobiology 47: 293-303.

BURDA H, SUMBERA R & BEGALL S (2007) Microclimate in burrows of subterranean rodents – revisited. In: BEGALL S, BURDA H & SCHLEICH CE (eds) Subterranean rodents: News from underground. Springer Verlag, Heidelberg, pp 21-33.

CAMPBELL CAM, PETTERSON J, PICKETT JA, WADHAMS L J & WOODCOCK CM (1993) Spring migration of damson hop aphid, *Phorodon humuli* (Homoptera, Aphididae) and summer host plant derived semiochemicals released on feeding. Journal of Chemical Ecology 19: 1569–1576.

CAVAGGIONI A & MUCIGNAT-CARETTA C (2000) Major urinary proteins, alpha(2u)-globulins and aphrodisin. Biochimica et Biophysica Acta – Protein Structure and Molecular Enzymology 1482: 218-228.

CAVAGGIONI A, MUCIGNAT-CARETTA C & ZAGOTTO G (2003) Absolute configuration of 2-sec-butyl-4,5-dihydrothiazole in male mouse urine. Chemical Senses 28: 791-797.

CHEETHAM SA, THOM MD, JURY F, OLLIER WER, BEYNON RJ & HURST JL (2007) The genetic basis of individual-recognition signals in the mouse. Current Biology 17: 1771-1777.

CLARKE FM & FAULKES CG (1999) Kin discrimination and female mate choice in the naked mole-rat *Heterocephalus glaber*. Proceedings of the Royal Society of London, Series B 266: 1995-2002.

CONAWAY CH (1971) Ecological adaptation and mammalian reproduction. Biology of Reproduction 4: 239-247.

DAWKINS R (1982) The extended phenotype. Freeman, San Francisco.

DEVRIES AC, JOHNSON, CL & CARTER CS (1997) Familiarity and gender influence social preferences in prairie voles (*Microtus ochrogaster*). Canadian Journal of Zoology 75: 295-301.

DAVIES JV & BELLAMY D (1972) The olfactory response of mice to urine and effects of gonadectomy. Journal of Endocrinology 55: 11-20.

DEMINE R & WALDEN P (2004) Sequit: software for de novo peptide sequencing by matrix-assised laser desorption/ionization post-source decay mass spectrometry. Rapid Communications in Mass Spectrometry 18: 907-913.

ERBILGIN N, GILLETTE N, MORI S, STEIN J, OWEN D & WOOD D (2007) Acetophenone as an anti-attractant for the Western Pine Beetle, *Dendroctonus Brevicomis*, LeConte (Coleoptera: Scolytidae). Journal of Chemical Ecology 33: 817-823.

FANJUL MS, ZENUTO RR & BUSCH C (2003) Use of olfaction for sexual recognition in the subterranean rodent *Ctenomys talarum*. Acta Theriologica 48: 35-46.

FAULKES CG, ABBOTT DH & JARVIS JU (1990) Social suppression of ovarian cyclicity in captive and wild colonies of naked mole-rats, *Heterocephalus glaber*. Journal of Reproduction and Fertility 88: 559-568.

FAULKES CG, VERHEYEN E, VERHEYEN W, JARVIS JUM, & BENNETT NC (2004) Phylogeographic pattern of specation and genetic divergence in African mole-rats (Family Bathyergidae). Molecular Ecology 13: 613-629.

FIRESTEIN S (2001) How the olfactory system makes a sense of scents. Nature 413: 211-218.

FLINT HM, SALTER SS, & WALTERS S (1979) Caryophyllene: An attractant for the greenlacewing. Environmental Entomology 8: 1123–1125.

FLOWER DR (1996) The lipocalin protein family: structure and function. Biochemical Journal 318: 1-14.

GIDLEY-BAIRD AA, WHITE BM, HAU J & POULSEN OM (1986) Initiation and control of ovulation in the mouse luteal phases. Effects of oestrogen, progesterone and prolactin. Acta Endocrinologica 113: 582-587.

GRAHAM JM & DESJARDINS C (1980) Classical conditioning: induction of luteinizing hormone and testosterone secretion in anticipation of sexual activity. Science 210: 1039-1041.

GREENWALD GS & ROTHCHILD I (1968) Formation and maintenance of corpora lutea in laboratory animals. Journal of Animal Science 27: 139-162.

GUERIN PM, STADLER E, & BUSER HR (1983) Identification of host plant attractants for the carrot fly, *Psila rosae*. Journal of Chemical Ecology 9: 843–861.

HAGEMEYER P & BEGALL S (2006) Individual odor similarity and discrimination in the coruro (*Spalacopus cyanus*, Octodontidae). Ethology 112: 529-536.

HAGEMEYER P, LANGE S, BREOCKER-PREUSS M & BURDA H (2009) The influence of olfactory stimulus and sexual activity on gonadal steroids in eusocial mole-rats. Folia Zoologica 58: 65-74.

HALLEM EA, FOX AN, ZWIEBEL LJ & CARLSON JR (2004) Olfaction: Mosquito receptor for human-sweat odorant. Nature 427: 212-213.

HALPERN M & MARTINEZ-MARCOS A (2003) Structure and function of the vomeronasal sytem: an update. Progress in Neurobiology 70: 245-318.

HALPIN ZT (1986) Individual odors among mammals: origins and functions. Advances in the Study of Behavior 16: 39-70.

HAMDAN MH & RIGHETTI PG (2005) Proteomics today. Protein assessment in biomarkers using mass spectrometry, 2D electrophoresis, and microarray technology (Wiley-Interscience Series in Mass Spectrometry). First edition. Wiley, AJ & Sons, Hoboken, New Jersey and Canada.

HAUPT A (2006): Identifikation von Proteinen anhand von MS/MS-Fragmentspektren. Sudienarbeit. Humbold-Universität zu Berlin, Berlin.

HARVEY S, JEMIOLO B & NOVOTNY M (1989) Pattern of volatile compounds in dominant and subordinate male mouse urine. Journal of Chemical Ecology 15: 2061-2072.

HENRY EC, DENGLER-CRISH CM, CATANIA KC (2007) Growing out of a caste – reproduction and the making of the queen mole-rat. Journal of Experimental Biology 210: 261-268.

HENZEL WJ, RODRIGUEZ H, SINGER AG, STUTS JT, MACRIDES F, AGOSTA WC & NIALL H (1988) The primary structure of aphrodisin. The Journal of Biological Chemistry 263: 16682-16687.

HETH G, BEAUCHAMP G, NEVO E & YAMAZAKI K (1996a) Species, population and individual specific odors in urine of mole rats (*Spalax ehrenbergi*) detected by laboratory rats. Chemoecology 7: 107-111.

HETH G, NEVO E & TODRANK J (1996b). Seasonal changes in urinary odors and in responses to them by blind suberranean mole rats. Physiology & Behaviour 60: 963-968.

HETH G & TODRANK J (2000): Individual odor similarities across species parallel phylogenetic relationships in the *S. ehrenbergi* superspecies of mole-rats. Animal Behaviour 60: 789-795.

HETH G, TODRANK J, BUSQUET N & BAUDOIN C (2001) Odor-genes covariance and differential investigation of individual odors in the *Mus* species complex. Biological Journal of the Linnean Society London 73: 213-220.

HETH G, TODRANK J & BURDA H (2002) Individual odor similarities within colonies and across species of eusocial *Cryptomys* mole rats. Journal of Mammalogy 83: 569-575.

HETH G, TODRANK J, BUSQUET N & BAUDOIN C (2003) Genetic relatedness assessment through individual odor similarities in mice. Biological Journal of the Linnean Society 78: 595-603.

HETH G, TODRANK J, BEGALL S, WEGNER R & BURDA H (2004) Genetic relatedness discrimination in an eusocial rodent, *Cryptomys anselli* mole-rats. Folia Zoologica 31: 997-1014.

HISAW F (1947) Development of the graafian follicle and ovulation. Physiological Reviews 27: 95-119.

HUMPHREYS EM, GHIONE R, GOSDEN RG, HOBSON BM & WIDE L (1985) Relationship between corpora lutea or fetal number on plasma concentrations of progesterone and testosterone in mice. Journal of Physiology London 75: 7-15.

HUMPHRIES RE, ROBERTSON DHL, BEYNON RJ & HURST JL (1999) Unravelling the chemical basis of competitive scent marking in house mice. Animal Behaviour 58: 1177-1190.

HURST JL, ROBERTSON DHL, TOLLADAY U & BEYNON RJ (1998) Proteins in urine scent marks of male house mice extend the longevity of olfactory signals. Animal Behaviour 55: 289-1297.

HURST JL, PAYNE CE, NEVISON CHM, MARIE AD, HUMPHRIES RE, ROBERTSON DHL, CAVAGGIONI A & BEYNON RJ (2001) Individual recognition in mice mediated by major urinary proteins. Nature 414: 631-633.

HURST JL & BEYNON RJ (2004) Scent wars: the chemobiology of competitive signalling in mice. Bioassays 26: 1288-1298.

HURST JL, THOM MD, NEVISON CM, HUMPHRIES RE & BEYNON R (2005). MHC odors are not required for recognition of individual scent owners. Proceedings of the Royal Society of London, Series B 272: 715-724.

INGRAM CM, BURDA H & HONEYCUTT RL (2004) Molecular phylogenetics and taxonomy of the African mole-rats, genus *Cryptomys* and the new genus *Coetomys* Gray, 1864. Molecular Phylogenetics and Evolution 31: 997-1014.

JANG T, SINGER AG & MACRIDES F (1995) Induction of c-fos-gene product in the male hamster accessory olfactory bulbs by natural and bacterially cloned aphrodisin. Chemical Senses 20: 125-125.

JANG TC, SINGER AG & O'CONNELL RJ (2001) Induction of c-fos in hamster accessory olfactory bulbs by natural and cloned aphrodisin. Neuroreport 3: 449-452.

JEMIOLO B, XIE TM & NOVOTNY M (1992) Urine marking in male mice: responses to natural and synthetic chemosignals. Physiolology & Behavior 52: 521-526.

JOHNSTON RE, & RASMUSSEN K (1984) Individual recognition of female hamsters by males: role of chemical cues and of the olfactory and vomeronasal systems. Physiolology & Behavior 33: 95-104.

JOHNSTON RE (2000) Chemical communication and pheromones: the types of signals and the role of the vomeronasal system. In: FINGER TE, SIVER WL & RESTREPO D (eds) The neurobiology of taste and smell. Second edition. John Wiley & Sons, New York, pp 10-127.

JOHNSTON RE (2003) Chemical communication in rodents: From pheromones to individual recognition. Journal of Mammalogy 84: 1141-1162.

KARLSON P & LUSCHER M (1959) Pheromones: a new term for a class of biologically active substances. Nature 183: 55-56.

KARLSON P & BUTENANDT A (1959) Pheromones (ectohormones) in insects. Annual Review of Entomology 4: 39-58.

KATZAV-GOZANSKY T (2006) The evolution of honeybee multiple queen-pheromones – A consequence of a queen-worker arms race? Brazilian Journal of Morphological Science 23: 287-294.

KAYALI-SAYADI MN, BAUTISTA JM, POLO-DIEZ LM & SALAZAR I (2003) Identification of pheromones in mouse urine by head-space solid-phase microextraction followed by gas chromatography-mass spectrometry. Journal of Chromatography B 796: 55-62.

KAYANJA FIB & JARVIS JUM (1971) Histological observations on the ovary, oviduct and unterus of the naked mole-rat. Zeitschrift für Säugetierkunde 36: 114-121.

KHALILOVA, AZ, PARAMONOV EA, ODINOKOV VN & KHALILOV LM (1998) Identification and biological activity of volatile organic substances emitted by plants and insects. 1. Components of the native scents of *Leptinotarsa decemlineata* and *Solanum tuberosum*. Chemistry of Natural Compounds 34: 647–649.

KOCK D, INGRAM CM, FRABOTTA LJ, HONEYCUTT RL & BURDA H (2006) On the nomenclature of Bathyergidae and *Fukomys* n. gen. (Mammalia: Rodentia). Zootaxa 1142: 51-55.

KRAMER B (2006) Ausgewählte endokrinologische Parameter im Urin von Graumullen (*Fukomys* spec.). Diplomarbeit im integrierten Studiengang Ökologie. Universität Duisburg-Essen, Essen.

LAI S-C & JOHNSTON RE (1994) The repertoire of individual odors in Djungarian hamsters, *Phodopus campbelli*. Ethology 96: 117-26.

LANGE S, NEUMANN B, HAGEMEYER P & BURDA H (2005) Kairomon guided food location in Zambian mole-rats (*Cryptomys spp.*, Bathyergidae). Folia Zoologica 54: 263-268.

LEHMAN-MCKEEMAN LD, CAUDILL D, RODRIGUEZ PA & EDDY C (1998): 2-sec-butyl-4,5-dihydrothiazole is a ligand for mouse urinary protein and rat alpha 2u-globulin: Physiological and toxicological relevance. Toxicology and Applied Pharmacology 149: 32-40.

LINTERN-MOORE S & PANTELOURIS EM (1975) Ovarian development in athymic nude mice. II. The growth of the oocyte and follicle. Mechanics of Ageing and Developement 4: 391-398.

LÜCKE CHR, FRANZONI L, ABBATE F, LÖHR F, FERRARI, E, SORBI RT, RÜTERJANS H & SPINSI A (1999) Solution structure of recombinant mouse major urinary protein. European Journal of Biochemistry 266: 1210-1218.

MA W, MIAO Z & NOVOTNY M (1999) Induction of estrus in grouped female mice (*Mus domesticus)* by synthetic analogues of preputial gland constituents. Chemical Senses 24: 289-293.

MACINNES JI, NOZIK ES & KURTZ DT (1986) Tissue-specific expression of the rat alpha-2u globulin gene family. Molecular and Cellular Biology 6: 3563-3567.

MAEDA K, OHKURA S & TSUKAMURA H (2000) Physiology of reproduction. In: KRINKE GJ (ed.) The Handbook of Experimental Animals - The Laboratory Rat. Academic Press, London, pp. 145-171.

MÄGERT HJ, HADRYS T, CIESLAK A, GROGER A, FELLER S & FORSSMANN WG (1995) cDNA sequence and expression pattern of the putative pheromone carrier aphrodisin. Proceedings of the National Academy of Sciences USA 92: 2091-2095.

MÄGERT HJ, CISLAK A, ALKAN O, LÜSCHER B, KAUFFELS W & FORSSMANN WG (1999) The golden hamster aphrodisin gene. Structure, expression in parotid glands of females animals, and comparison with a similar murine gene. Journal of Biological Chemistry 274: 444-450.

MALHERBE G (2004) Is the highveld mole-rat *Cryptomys hottentotus pretoriae* (Rodentia, Bathyergidae) an induced or spontaneous ovulator? Zoological Journal of the Linnean Society 263: 159-165.

MARIE AD, VEGGERBY C, ROBERTSON DHL, GASKELL SJ, HUBBARD SJ, MARTINSEN L, HURST JL & BEYNON RJ (2001) Effect of polymorphisms on ligand binding by mouse major urinary proteins. Protein Science 10: 411-417.

MARTÌNEZ-RICÒS J, AGUSTÌN-PAVÒN C, LANUZA E & MARTÌNEZ-GARCIA F (2007) Intraspecific communication through cemical signals in female mice: Reinforcing properties of involatile male sexual pheromones. Chemical Senses 32: 139-148.

MATEO JM & JOHNSTON RE (2000) Kin recognition and the "armpit effect": evidence of self-referent phenotype matching: Proceedings of the Royal Society of London, Series B 267: 695-700.

MATEO JM (2003) Kin recognition in ground squirrels and other rodents. Journal of Mammalogy 84: 1163-1181.

McFadyen DA & Locke J (2000) High-resolution FISH mapping of the rat alpha(2u)-globulin multigene family. Mammalian Genome 11: 292-299.

McGee EA & Hsueh AJW (2000) Initial and cyclic recruitment of ovarian follicles. Endocrine Reviews 21: 200-214.

Menzies RA, Heth G, Ikan R, Weinstein V & Nevo E (1992) Sexual pheromones in lipids and other fractions from urine of the male mole rat, *Spalax ehrenbergi*. Physiology & Behavior 52: 741-747.

Michael RP & Keverne EB (1968) Pheromones in the communication of sexual status in primates. Nature 218: 746-749.

Miller BH, Olson SL, Turek FW, Levine JE, Horton TH & Takahashi JS (2004) Circadian clock mutation disrupts estrous cyclicity and maintenance of pregnancy. Current Biology 14: 1367-1373.

Molteno A & Bennett NC (2000) Anovulation in non-reproductive female Damaraland mole-rats (*Cryptomys damarensis*). Journal of Reproduction and Fertility 119: 35-41.

Mondain-Monval M, Dutourne B, Bonnin-Laffargue M, Canivenc R & Scholler R (1977) Ovarian activity during the anoestrus and the reproductive season of the red fox (*Vulpes Vulpes L.*). Journal of Steroid Biochemistry 8: 761-769.

Mucignat-Caretta C, Caretta A & Cavaggioni A (1995) Acceleration of puberty onset in female mice by male urinary proteins. Journal of Physiology 486: 517-522.

Mucignat-Caretta C, Colivicchi MA, Fattori M, Ballini C, Bianchi L, Gabai G, Cavaggioni A & Della Corte L (2006) Species-specific chemosignals evoke delayed excitation of the vomeronasal amygdala in freely-moving female rats. Journal of Neurochemistry 99: 881-891.

Munclinger P & Frynta D (2000) Social interactions within and between two distant populations of house mouse. Folia Zoologica 49: 1-6.

Murr SM, Geschwind II & Bradford GE (1973) Plasma LH and FSH during different estrous-cycle conditions in mice. Journal of Reproduction and Fertility 32: 221-230.

Nelson JF, Felicio LS, Randall PK, Sims C & Finch CE (1982) A longitudinal study of estrous cyclicity in aging C57BL/6J mice: I. Cycle frequency, length and vaginal cytology. Biology of Reproduction 27: 327-339.

Nevison CM, Armstong S, Beynon RJ, Humphries RE & Hurst JL (2003) The ownership signature in mouse scent marks is involatile. Proceedings of the Royal Society of London, Series B 270: 1957-1963.

NESVIZHSKII AI (2006) Potein identification by tandem mass spectrometry and sequence database searching. In: MATTIESEN R (ed) Mass spectrometry data analysis in proteomics. Erste Auflage, Springer Verlag, Berlin, pp 90-91.

NEVO E, HETH G, AUFFRAY JC & BEILES A (1987) Preliminary studies of sex pheromones in the urine of actively speciating subterranean mole rats of the *Spalax ehrenbregi* superspcies in Israel. Phytoparasitica 15: 163-164.

NEVO E, IVANITSKAYA E & BEILES A (2001) Adaptive radiation of blind subterranean mole rats: Naming and revisiting the four sibling species of the *Spalax ehrenbergi* superspecies in Israel: *Spalax galili* (2n=52), *S. golani* (2n=54), *S. carmeli* (2n=58), and *S. judaei* (2n=60). Backhuys Publishers, Leiden.

NORDLUND DA & LEWIS WJ (1976) Terminology of chemical releasing stimuli in intraspecific and interspecific interactions. Journal of Chemical Ecology 2: 211-220.

NOVOTNY M, HARVEY S, JEMIOLO B & ALBERTS J (1984) Synthetic pheromones that promote inter-male aggression in mice. Proceedings of the National Academy of Sciences USA 82: 2059-2061.

NOVOTNY M, JEMIOLO B, HARVEY S, WIESLER D & MARCHLEWSKA-KOJ A (1986) Adrenal-mediated endogenous metabolites inhibit puberty in female mice. Science 231: 722-725.

NOVOTNY M, HARVEY S & JEMIOLO B (1990) Chemistry of male dominance in the house mouse, *Mus domesticus*. Experientia 46: 109-113.

NOVOTNY M, JEMIOLO B, WIESLER D, WEIDONG M, SCOTT H, XU F, XIE TM & CARMACK M (1999a) A unique urinary constituent, 6-hydroxy-6-methyl-3-heptanone, is a pheromone that accelerates puberty in female mice. Chemistry and Biology 6: 377-383.

NOVOTNY M, WEIDONG M, WIESLER D & ZIDEK L (1999b) Positive identification of the puberty-accelerating pheromone of the house mouse: The volatile ligands associating with the major urinary protein. Proceedings of the Royal Society of London, Series B: Biological Sciences 266: 2017-2022.

PEELE P, SALAZAR I, MIMMACK M, KEVERNE EB & BRENNAN PA (2003) Low molecular weight constituents of male mouse urine mediate the pregnancy block effect and convey information about the identity of the mating male. European Journal of Neuroscience 18: 622-628.

PELOSI P (2001) The role of perireceptor events in vertebrate olfaction. Cellular and Molecular Life Sciences 58: 503-509.

PINO JA, MESA J, MUNOZ Y, MARTI MP & MARBOT R (2005) Volatile components from mango (*Mangifera indica L.*) cultivars. Journal of Agriculture and Food Chemistry 53: 2213-2223.

RASMUSSEN LEL, LEE TD, OOELOFS WL, ZHANG AJ & DAVES GD (1996) Insect pheromone in elephants. Nature 415: 975-976.

RASMUSSEN LEL & PERRIN TE (1999) Physiological correlates of musth: Lipid metabolites and chemical composition of exudates. Physiology & Behavior 67: 539-549.

RASMUSSEN LEL & WITTEMYER G (2002) Chemosignalling of musth by individual wild African elephants (Loxodonta africana): implications for conservation and management. Proceedings of the Royal Society of London, Series B 269: 853-860.

REASNER DS & JOHNSTON RE (1988) Acceleration of reproductive development in female Djungarian Hamsters by adult males. Physiology & Behavior 43: 57-64.

REGNIER FE (1971) Semiochemicals - structure and function. Biology of Reproduction 4: 309-326.

ROBERTSON DHL, BEYNON RJ & EVERSHED RP (1993) Extraction, characterisation and binding analysis of two pheromonally active ligands associated with major urinary protein of house mouse (*Mus musculus*). Journal of Chemical Ecology 19: 1405-1417.

ROBERTSON DHL, COX KA, GASKELL SJ, EVERSHED RP & BEYNON RJ (1996) Molecular heterogeneity in the major urinary proteins of the house mouse *Mus musculus*. Biochemical Journal 316: 265-272.

ROBERTSON DHL, HURST JL, SEARLE JB, GÜNDÜZ I & BEYNON RJ (2007) Characterization and comparison of major urinary proteins from the house mouse, *Mus musculus domesticus*, and the aboriginal mouse, *Mus macedonicus*. Journal of Chemical Ecology 33: 613-630.

RUSTOM R, COSTIGAN M, SHENKIN A & BONE JM (1998) Proteinuria and renal tubular damage: Urinary N-acetyl-beta-D-glucosaminidase and isoenzymes in dissimilar renal disease. American Journal of Nephrology 18: 179-185.

SCHMIDT-NIELSON K (1997) Animals physiology: adaptation and environment. Fifth edition. Cambridge University Press, Cambridge UK.

SCHOECH SJ, MUMME R & WINGFIELD JC (1996) Delayed breeding in the cooperatively breeding Florida scrub-jay (*Aphelocoma coerulenscens*): inhibition or the absence of stimulation? Behavioral Ecology and Sociobiolgy 39: 77-90.

SCHWAB TM, SOLOMON NG, ISAACSON LG & CALLAHAN P (2004) Reproductive activation of pine voles (*Microtus pinetorum*): examination of physiological markers. Brain Research 1021: 256-263.

SCHWANZ LE & LACEY EA (2003) Olfactory discrimination of gender by colonial tuco-tucos (*Ctenomys sociabilis*). Mammalian Biology 68: 53-60.

SCHWENDE FJ, WIESLER D, JORGENSON JW, CARMACK M & NOVOTNY M (1985) Urinary volatile constituents of the house mouse, *Mus musculus*, and their endocrine dependency. Journal of Chemical Ecology 12: 277-296.

SHANAS U & TERKEL J (1996) Grooming secretions and seasonal adaptations in the blind mole rat (*Spalax ehrenbergi*). Physiology & Behavior 60: 653-56.

SHERBORNE AL, THOM MD, PATERSON S, JURY F, OLLIER WER, STOCKLEY P, BEYNON RJ & HURST JL (2007) The genetic basis of inbreeding avoidance in house mice. Current Biology 17: 2061-2066.

SIMON JE & QUINN J (1988) Characterization of essential oil of parsley. Journal of Agriculture and Food Chemistry 36: 467–472.

SINGER AG, AGOSTA WC & CLANCY AN (1987) The chemistry of vomeronasally detected pheromones: characterization of an aphrodisin protein. Annals of the New York Academy of Sciences 519: 287-298.

SINGER AG (1991) A chemistry of mammalian pheromones. Journal of Steroid Biochemistry and Molecular Biology 39: 627-632.

SOINI HA, WIESLER D, APFELBACH R, KÖNIG P, VASILIEVA NY & NOVOTNY MV (2005a) Comparative investigation of the volatile urinary profiles in different Phodopus hamster species. Journal of Chemical Ecology 31: 1125-1143.

SOINI HA, BRUCE KE, WIESLER D, DAVID F, SANDRA P & NOVOTNY MV (2005b) Stir bar sorbtive extraction: A new quantitative and comprehensive sampling technique for determination of chemical signal profiles from biological media. Journal of Chemical Ecology 21: 377-392.

SOLOMON NG, VANDENBERGH JG, WEKESA KS & BARGHUSEN L (1996) Chemical cues are necessary but insufficient for reproductive activation of female pine vole (*Microtus pinetorum*). Biology of Reproduction 54: 1038-1045.

SORENSEN PW & STACEY NE (1999) Evolution and specialization of fish hormonal pheromones. In: JOHNSTON RE, MÜLLER-SCHWARZE D & SORENSON PW (eds) Advances in chemical signals in vertebrates. Kluwer Academic Publishers/Plenum Press. New York, pp. 15-48.

SNYMAN PC, JACKSON CR & BENNETT NC (2006) Do dispersing non-reproductive female Damaraland mole-rats, *Cryptomys damarensis* (Rodentia: Bathyergidae) exhibit spontaneous or induced ovulation? Phyisology & Behavior 87: 88-94.

STERN K & MCCLINTOCK MK (1998) Regulation of ovulation by human pheromones. Nature 392: 177-179.

STOPKOVA R, STOPKA P, JANOTOVA K & JEDELSKÝ P (2007) Species-specific expression of major urinary proteins in the house mice (*Mus musculus musculus* and *Mus musculus domesticus*). Journal of Chemical Ecology 33: 861-869.

TANG-MARTINEZ Z, MUELLER LL & TAYLOR GT (1993) Individual odors and mating success in the golden hamster *Mesocricetus auratus*. Animal Behaviour 45: 1145-1151.

TODRANK J & HETH G (1996) Individual odors in two chromosomal species of blind subterranean mole rats (*Spalax ehrenbergi*): Conspecific and cross-species discrimination. Ethology 102: 806-811.

TODRANK J, HETH G & JOHNSTON RE (1998) Kin recognition in golden hamsters: evidence for kinship odors. Animal Behavior 55: 377-386.

TODRANK J, HETH G & JOHNSTON RE (1999) Social interaction is necessary for discrimination between and memory for odors of close relatives in golden hamsters. Ethology 105: 771-782.

TODRANK J & HETH G (2003) Odor-genes covariance and genetic relatedness assessments: Rethinking odor-based "recognition" mechanisms in rodents. Advances in the Study of Behavior 32: 77-130.

TRAURIG HH (1970) Epithel cell proliferation in the mouse vagina during estrus cycle, pregnancy and lactation. Anatomical Record 170: 457-470.

VAN DEALE PAAG, DAMMANN P, KAWALIKA M, MEIER JL, VAN DE WOESTIJNE C & BURDA H (2004) Chromosomal diversity in *Chryptomys* mole-rats (Rodentia: Bathyergidae) in Zambia with the description of new karyotypes. Journal of the Proceedings of the Linean Society 264: 317-326.

VANDENBERGH JG (1969) Male odor accelerates female sexual maturation in mice. Endocrinology 84: 658-660.

VAN RENSBURG LJ, BENNETT NC, VAN DER MERWE M, SCHOEMAN AS & BRINDERS J (2003) Are non-reproductive male highveld mole-rats, *Cryptomys hottentotus pretoriae* physiologically suppressed while in the confines of the natal colony? Journal of Zoology 260: 73-78.

VINCENT F, LOBEL D, BROWN K, SPINELLI S, GROTE P, BREER H, CAMBILLAU C & TEGONI M (2001) Crystal structure of aphrodisin, a sex pheromon from female hamster. Journal of Molecular Biolology 305: 459-469.

WANG H & DEY SK (2006) The window of uterine receptivity in mice and humans. Nature Reviews Genetics 7: 185-199.

WALDMAN B, FRUMHOFF PC & SHERMAN PW (1988) Problems of kin recognition. Trends Ecology and Evolution 3: 8-13.

WALMER DK, WRONA MA, HUGES CL & NELSON KG (1992) Lactoferrin expression in the mouse reproductive tract during the natural estrus cycle: correlation with circulating estradiol and progesterone. Endocrinology 131: 1458-1466.

WEBER J (2003) Entwicklung zur Festphasenmikroextraction für flüchtige Verbindungen unter Einsatz der Gaschromatographie/Massenspektrometrie. PhD thesis, Technische Universität Clausthal, Clausthal Germany.

WEISSTEINER S & SCHÜTZ S (2006) Are different pattern influencing host plant choice of belowground living insects? Mitteilungen der Deutschen Gesellschaft für Allgemeine und Angewandte Entomologie 15: 51-55.

WERSINGER SR & RISSMAN EF (2000) Oestrogen receptor alpha is essential for female-directed chemo-investigatory behaviour but is not required for the pheromone-induced luteinizing hormone surge in male mice. Journal of Neuroendocrinology 12: 103-110.

WESTLIN L, BENNETT NC & JARVIS JUM (1994) Relaxation of reproductive suppression in non-breeding naked mole-rats. Journal of the Linnean Society 234: 177-188.

WILLIAMS JR, SLOTNICK BM, KIRKPATRICK BW & CARTER CS (1992) Olfactory-bulb removal affects partner preference development and estrus induction in female Prairie Voles. Physiology & Behavior 52: 635-639.

WILSON EO & BOSSERT WH (1963) Chemical communicaton among animals. Recent Progress in Hormone Research 19: 673-716.

WILLSE A, KWAK J, YAMAZAKI K, PRETI G, WAHL JH & BEAUCHAMP GK (2006) Individual odor types; interaction of MHC and background genes. Immunogenetics 58: 967-982.

WILLINGSTORFER WJ, BURDA H & WINCKLER J (1998) Ovarian growth and folliculogenesis in breeding and nonbreeding females of a social rodent, the Zambian common mole-rat, *Cryptomys* sp. Journal of Morphology 237: 33-41.

WYATT TD (2003) Pheromones and Animal Behaviour. First edition. The Press Syndicate of the University of Cambridge, Cambridge, UK.

XU F, SCHAEFER M, KIDA I, SCHAFER J, LIU N, ROTHMAN DL, HYDER F, RESTREPO D & SHEPHERD GM (2005) Simultaneous activation of mouse main and accessory olfactory bulbs by odors or pheromones. Journal of Comparative Neurology 489: 491-500.

ZHANG JX, SOINI HA, BRUCE KE, WIESLER D, WOODLEY SK, BAUM MJ & NOVOTNY MV (2005) Putative Chemosignals of the ferret (*Mustela furo*) associated with individual and gender recognition. Chemical Senses 30: 727-737.

ZHANG JX, RAO XP, SUN L, ZHAO CHH & QIN XW (2007) Putative chemical signal about sex, individuality, and genetic background in the preputial gland and urine of the house mouse (*Mus musculus*). Chemical Senses 32: 293-303.

ZHANG JX, SUN L, ZHANG JH & FENG ZY (2008) Sex- and gonad-affecting scent compounds and 3 male pheromones in the rat. Chemical Senses 33: 611–621.

ZENUTO RR & FANJUL SM (2002) Olfactory discrimination of individual scents in the subterranean rodent *Ctenomys talarum* (tuco-tuco). Ethology 108: 629-641.

ZIDEK L (1999) Pheromone-binding proteins in the house mouse. PhD-thesis, Indiana University Indiana, Department of Chemistry.

References (on-line):

URL1: **OVERHISER A** (1999) Solution structure of MUP. Access on 15.05.2002
http://www.chem.indiana.edu/biochemistry/biophygif.htm

URL2: **FIRESTEIN S** (2001). Acces on 20.7.2009
http://www.colorado.edu/intphys/iphy4480tsai/olfanatomy.jpg

URL3: **WANG & DEY** (2006). Access on 27.7.2009
http://www.nature.com/nrg/journal/v7/n3/images/nrg1808-i2.jpg

URL4: **SIGMA-ALDRICH** (2008). Access on 22.1.2008
http://www.sigmaaldrich.com/catalog/search/ProductDetail/SUPELCO/57328-U

URL5: **BLAST**. Access on 15.7.2009
http://blast.ncbi.nlm.nih.gov/Blast.cgi

VII APPENDIX

VII.A List of abbreviations

A	absorbance	**HPLC**	high pressure liquid chromatography
ANOVA	analysis of variance	**HS**	head space
BLAST	Basic Local Alignment Search Tool	**Hz**	hertz
° C	degree Celsius	**ID**	internal diameter
CHAPS	3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate	**i.a.**	*inter alia* (amongst others)
CI	confidence interval	**i.e.**	*id est* (that is)
Cr	creatinine	**IEF**	isoelectric focussing
C_{st}	concentration of standard	**IPG**	immobilized pH gradient
CW	carbowax	**IS**	internal standard
D	dimension	**kDa**	kilodalton
ddH_2O	double deionised water	**kV**	kilovolt
DTT	dithiotreithol	**l**	liter
DVB	divinlybenzene	**LH**	luteinizing hormone
e.g.	*examplia gratia* (for instance)	**M**	mol
EtOH	ethanol	**MALDI**	matrix-assisted-laser-desorption-ionization
F	female	**mg**	miligram
FA	*Fukomys anselli*	**min**	minute
fig.	figure	**ml**	mililitre
FK	*Fukomys kafuensis*	**mM**	milimol
FKA	*Fukomys kafuensis* × *anselli* (hybrids)	**MS**	mass spectrometry
GC	gas chromatography	**MUP**	major urinary protein
H	habituation	**N**	number
h	hour	**NCBI**	National Centre for Biotechnology Information
HCl	hydrogen chloride	**ND: YAG**	Neodymium-doped: Yttrium-aluminium-garnet laser
HP	Hewlett Packard	**ng**	nanogram

NIST	National Institute of Standards and Technology	**SBSE**	stir bar sorptive extraction
nmol	nanomol	**SD**	standard deviation
nrf	non-reproductive female	**SDS**	sodium dodecylsulfate
nrK	non-reproductive king	**sp.**	species (*singular*)
nrm	non-reproductive male	**spp.**	species (*plural*)
nrQ	non-reproductive queen	**SPME**	solid-phase microextraction
ns	not significant	**T**	test
NSA	not sexual active	**tab.**	table
PDMS	polydimethylsiloxane	**TOF**	time of flight
p	probability	**TIC**	total ion chromatogram
PA	peak area	**Tris**	tris(hydroxymethyl)aminomethane
PBS	phosphate buffered saline solution	**Vh**	volt hours
PDMS	polydimethylsiloxane	**v/v**	volume percent
pI	isoelectric point	**µA**	microampere
PMF	peptide mass fingerprint	**µmol**	micromol
PTFE	polytetraflourethylene	*	$p<0.05$, probability of error lower than 5%
Q	queen	**	$p<0.005$, probability of error lower than 1%
rK	reproductive king	***	$p<0.005$, probability of error lower than 0.1%
rpm	rounds per minute	♀	female
rQ	reproductive queen	♂	male
RT	retention time (min)	#	animal number
s	seconds		

VII.B Figure legends

Fig. VII.A.1: Collection of odours from the ano-genital region onto a glass plate (photo: S. Peuckmann).

Fig. VII.B1a-f: Nucleated cells in vaginal smear in different stages.

Fig. VII.B2a-f: Cornified cells in vaginal smear in different stages.

Fig. VII.B3a-f: Granulocytes in vaginal smear in different stages.

Fig. VII.B4-7: Pattern of two cell types (nucleated cells and cornified cells) of vaginal smear in relation to urinary progesterone concentrations (ng/mg Cr) in females of different categories. Females rQ1 and nrf1 were involved during the first test-phases and female nrQ1 and nrf2 were involved test phase one to three.

Fig. VII.B8: Urinary estradiol and progesterone concentrations of rQ5 in relation to nucleated cells, cornified cells and granulocytes measured during consecutive test-phases. Test phase one without manipulation until day 39;. ····· start of the 2nd test phase (olfactory stimulation), —— start of the 3rd test phase (mating).

Fig. VII.B9: Regression curve of three times measured estradiol (nmol/l). Means of estradiol concentrations showing approximate linearity. The dilution series was 1: 5, 1:10 and 1: 20.

Fig. VII.B10: Regression curve of three times measured progesterone (ng/ml). Means of estradiol concentrations showing approximate linearity. The dilution series was 1: 5, 1:10, 1: 20 and 1:50.

Fig. VII.C1: Blank of TIC (total ion chromatogram) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from individual nrf B of family one of an Ansell's *Fukomys* mole-rat (without solvent delay) illustrating clean fibre of the SPME-syringe and clean column of the gas chromatograph.

Fig. VII.C2: Regression curve of vertinpropionate (three measures per each dilution) by HS-SPME-GS-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry). Means of TIC (total ion chromatogram) show approximate linearity. The dilution series was 1: 2,000; 1:20,000 and 1: 200,000.

Fig. VII.C3: Regression curve of 1-(S)-α-pinene (three measures per each dilution) by HS-SPME-GS-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry). Means of TIC (total ion chromatogram) show approximate linearity. The dilution series was 1: 1,000; 1: 5,000; 1: 10,000.

Fig. VII.C4: TIC (total ion chromatogram) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry). Peaks are of 1-(S)-α-pinene and vertinpropionatee in ddH_2O, illustrating appropriateness as calibration compounds.

Fig. VII.C5: Validation of the retention time (RT in min) of the internal standards 1-(S)-α–pinene and vertinpropionate and their mean values of several GC-MS-measures, illustrating small error bars (with 95% lower and upper confidence intervals, CI; $\alpha = 0.05$) left and standard deviation (SD) right.

Fig. VII.C6a: TIC (total ion chromatogram) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of dd H_2O-sample. The mass spectrum of vertinpropionate (2-methoxy-3-(1-methylethyl)-pyrazine) below demonstrates a match of 935 and a probability of 92.5 % (NIST 2002), thus a high similarity of vertinpropionate to its reference (NIST 2002). Dilution of vertinpropionate: 1 µl (1: 2,000 EtOH) in 1,800 µl H_2O (RT = 33.38 min). RT = retention time (min).

Fig. VII.C6b: TIC (total ion chromatogram) of HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a dd H_2O-sample. The mass spectrum of 1-(S)-α-pinene below demonstrates a match of 918 (NIST 2002), thus a high similarity of 1-(S)-α-pinene to its reference (NIST 2002). Dilution of 1-(S)-α-pinene: 1 µl (1: 1,000 ethanol) in 1,800 µl H_2O (RT = 15.20 min). RT = retention time (min).

Fig. VII.C7a: TICs (total ion chromatograms) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from family four of hybrid *Fukomys* mole-rats. Peaks are with and without internal standards (IS) 1-(S)-α-pinene and vertinpropionate, illustrating their appropriateness and the masking effect of urinary peak at RT = 33.4 min by vertinpropionate.

Fig. VII.C7b: TICs (total ion chromatograms) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from the queen (Q) of family one of an Ansell's *Fukomys* mole-rat. Peaks are with and without the internal standard (IS) vertinpropionate, illustrating their appropriateness and the masking effect of urinary peak at RT = 33.4 min by vertinpropionate.

Fig. VII.C7c: TICs (total ion chromatograms) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from the queen (Q) of family three of an Ansell's *Fukomys* mole-rat. Peaks are with and without the internal standard (IS) vertinpropionate, illustrating their appropriateness and the masking effect of urinary peak at RT = 33.4 min by vertinpropionate.

Fig. VII.C8: TICs (total ion chromatograms) of two measures obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from family two of Ansell's *Fukomys* mole-rats during potato diet, illustrating similarity of repeated measures.

Fig. VII.C9: TICs (total ion chromatograms) of two measures obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from family two of Ansell's *Fukomys* mole-rats during carrot diet, illustrating similarity of repeated measures.

Fig. VII.C10: TIC (total ion chromatogram) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a dd H_2O-sample illustrating validation of the retention time (RT in min) of peak 23 (ß-caryophyllene) in the urinary volatiles of mole-rats (extra peaks are dirt of the standard substance). The mass spectrum below demonstrates identification of ß-caryophyllene with a match of 928 (NIST 2002).

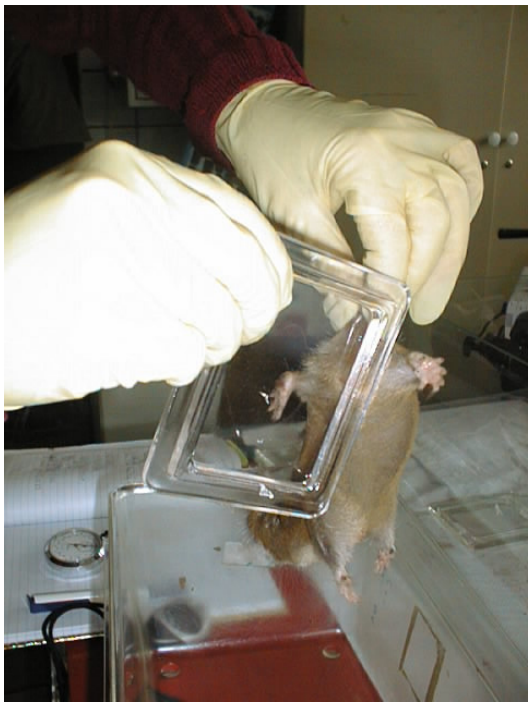
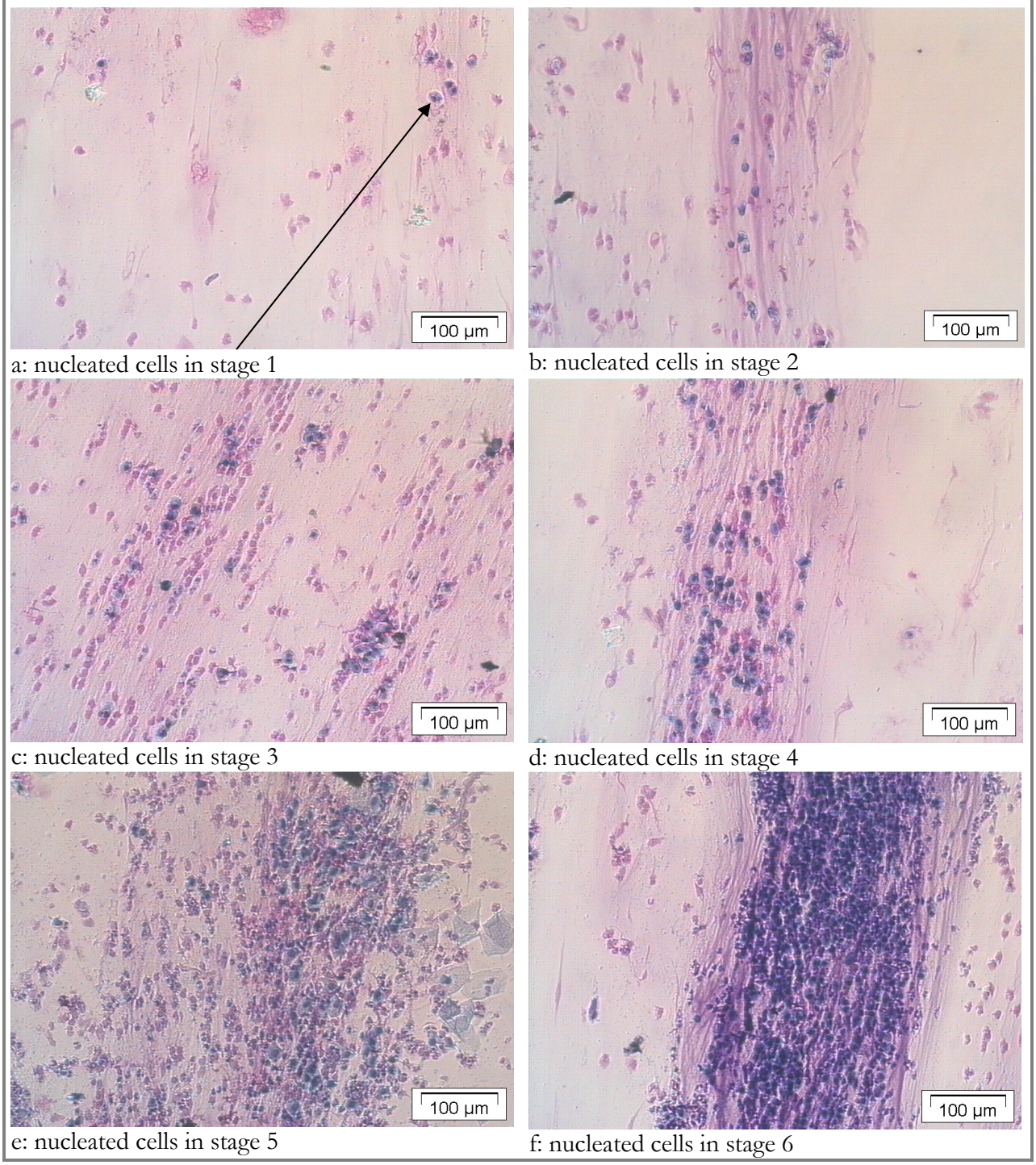
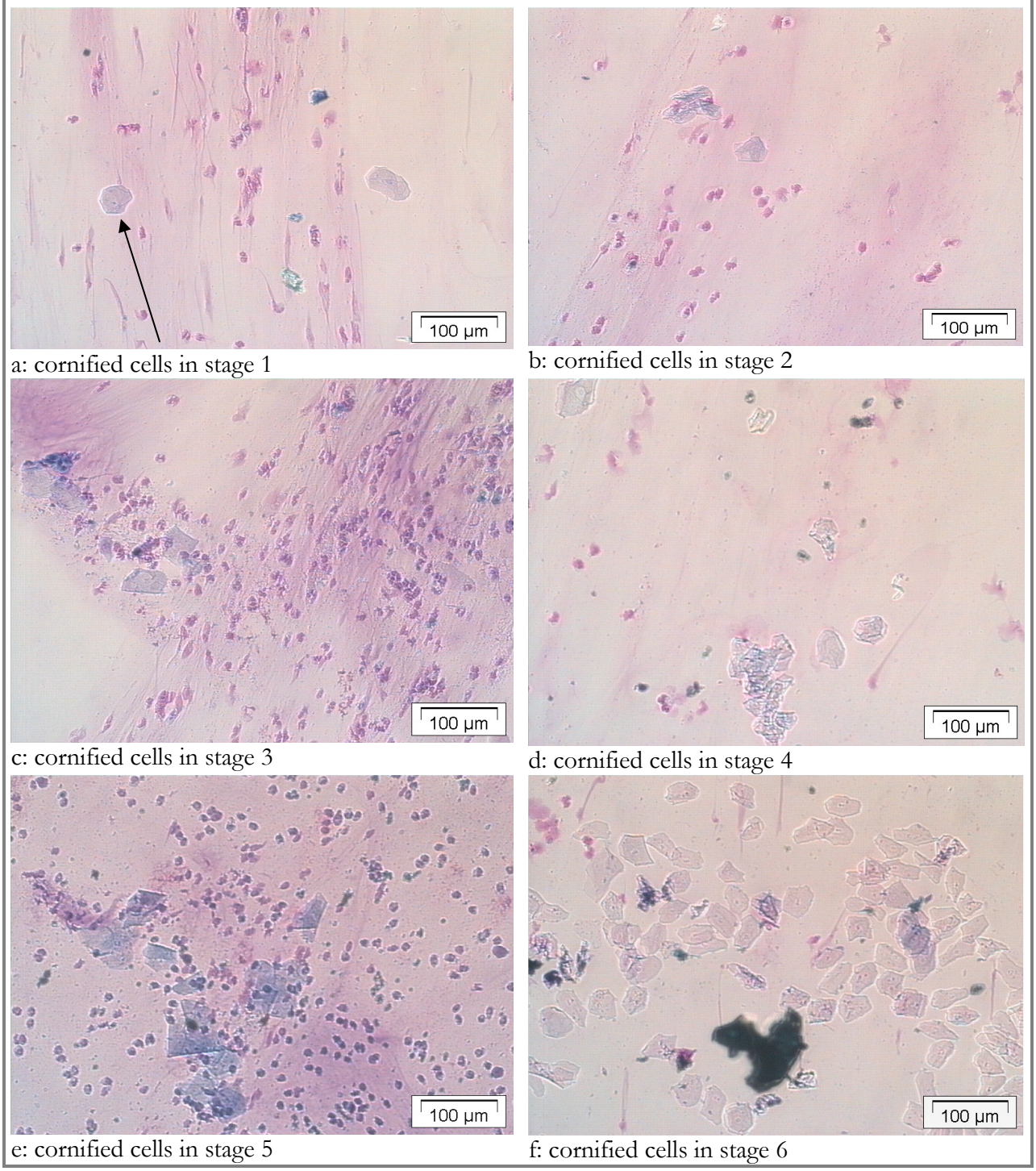


Fig. VII.A.1: Collection of odours from the ano-genital region onto a glass plate (photo by S. Peuckmann).



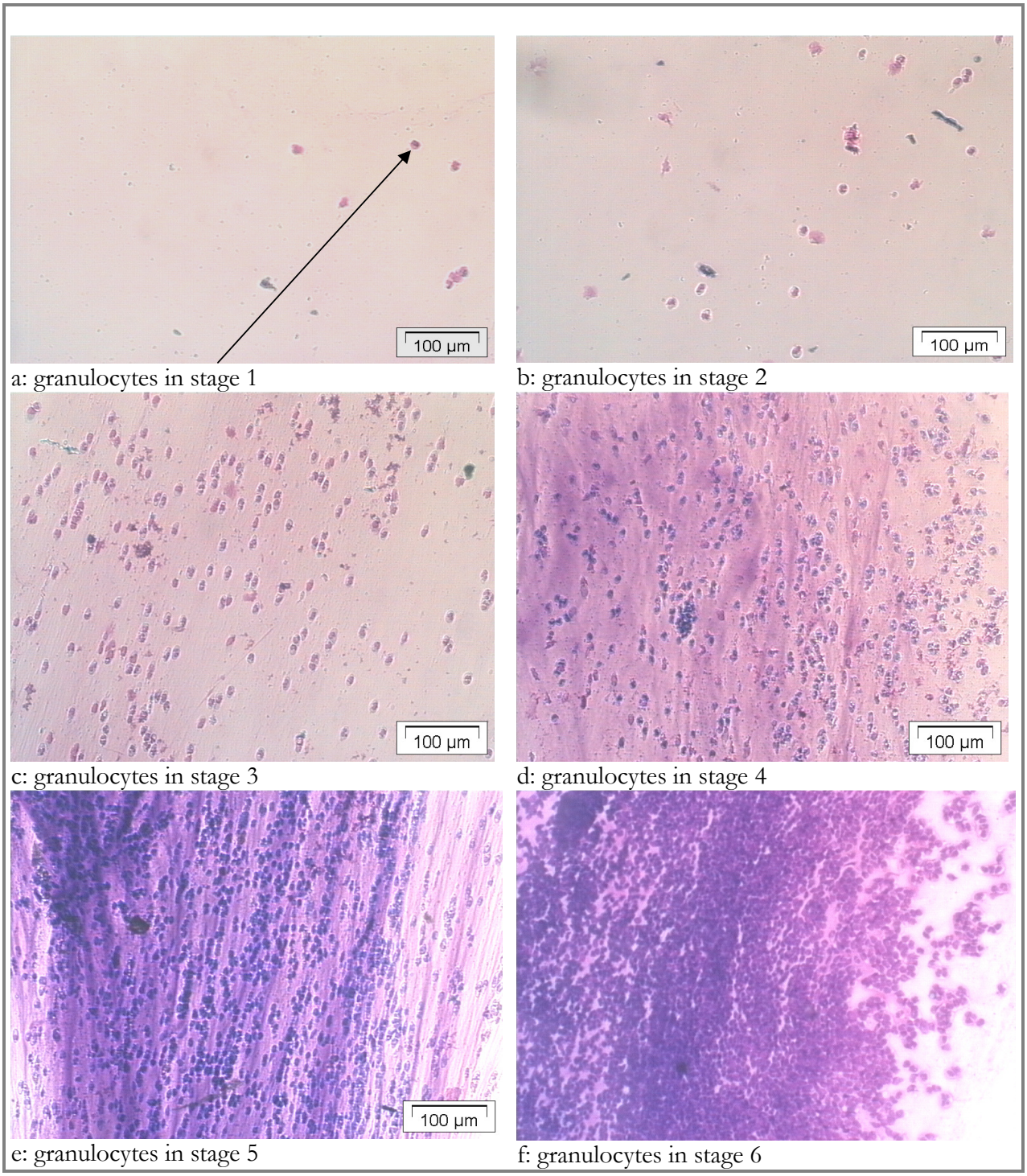
a: nucleated cells in stage 1
b: nucleated cells in stage 2
c: nucleated cells in stage 3
d: nucleated cells in stage 4
e: nucleated cells in stage 5
f: nucleated cells in stage 6

Fig. VII.B1a-f: Nucleated cells in vaginal smear in different stages.



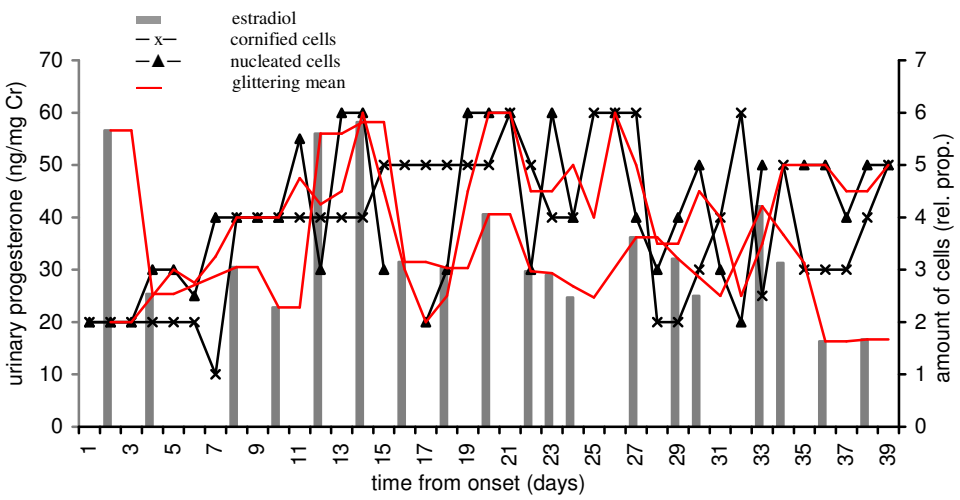
a: cornified cells in stage 1
b: cornified cells in stage 2
c: cornified cells in stage 3
d: cornified cells in stage 4
e: cornified cells in stage 5
f: cornified cells in stage 6

Fig. VII.B2a-f: Cornified cells in vaginal smear in different stages.

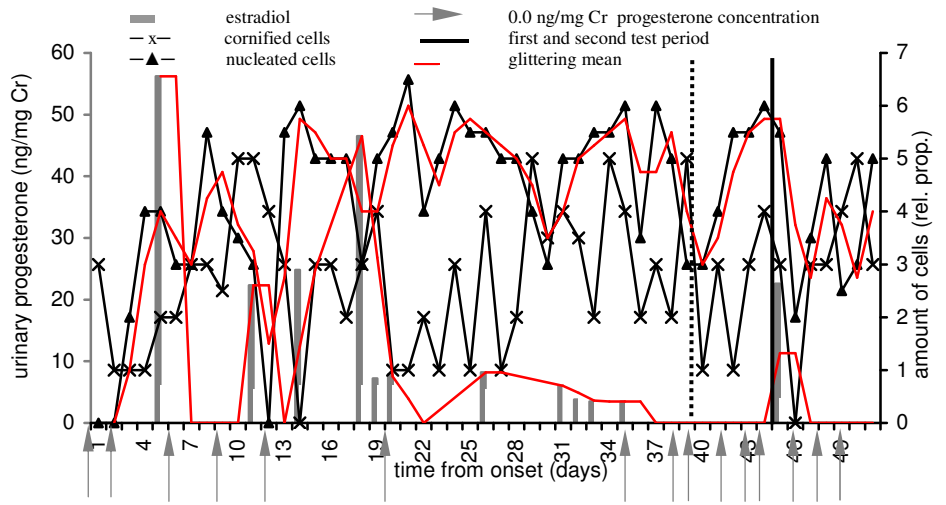


a: granulocytes in stage 1
b: granulocytes in stage 2
c: granulocytes in stage 3
d: granulocytes in stage 4
e: granulocytes in stage 5
f: granulocytes in stage 6

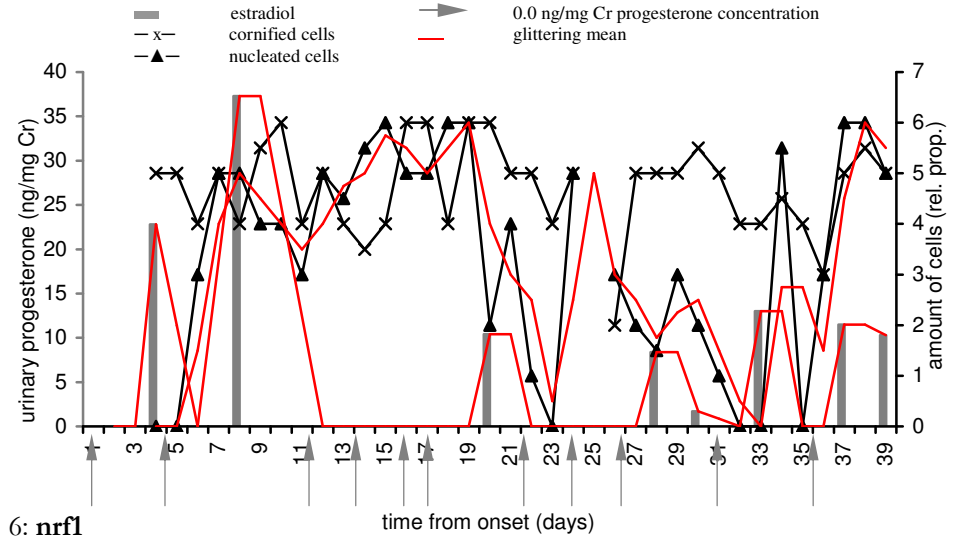
Fig. VII.B3a-f: Granulocytes in vaginal smear in different stages.



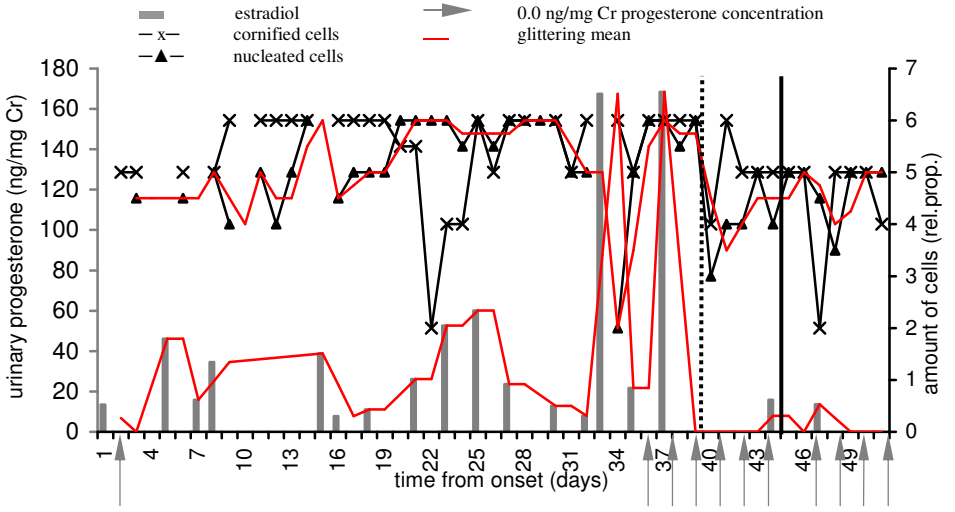
4: **rQ2**



5: **nrQ1**



6: **nrf1**



7: **nrf2**

Fig. VII.B4-7: Pattern of two cell types (nucleated cells and cornified cells) of vaginal smear in relation to **urinary progesterone concentrations** (ng/mg Cr) in females of different categories. Females rQ1 and nrf1 were involved the first test-phase and female nrQ1 and nrf2 were involved in the test-phases one to three.

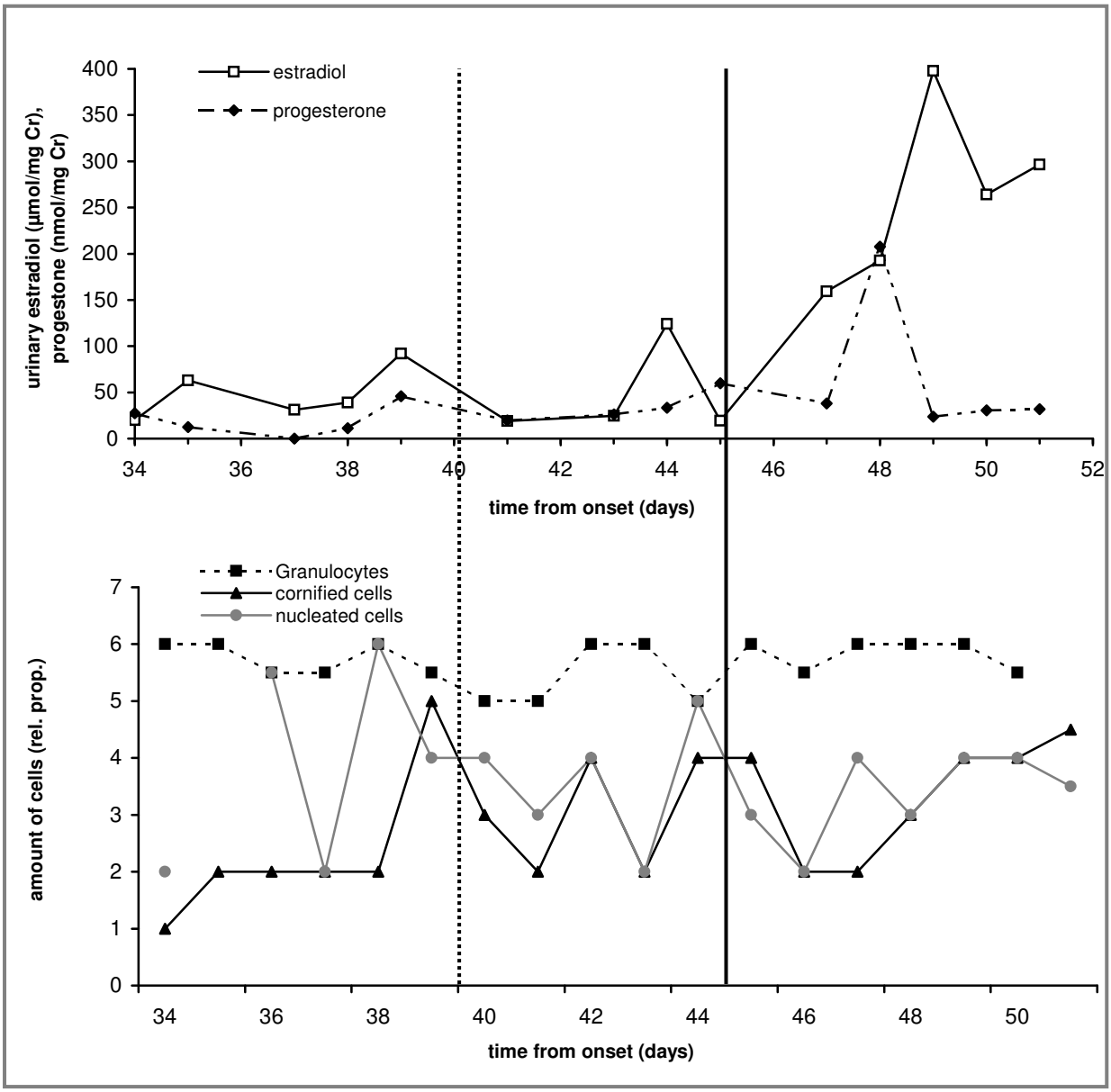


Fig. VII.B8: Urinary estradiol and **progesterone concentrations** of **rQ5** in relation to **nucleated cells**, **cornified cells** and **granulocytes** measured during consecutive test-phases. Test phase one without manipulation until day 39;. ····· start of the 2nd test phase (olfactory stimulation), ——— start of the 3rd test phase (mating).

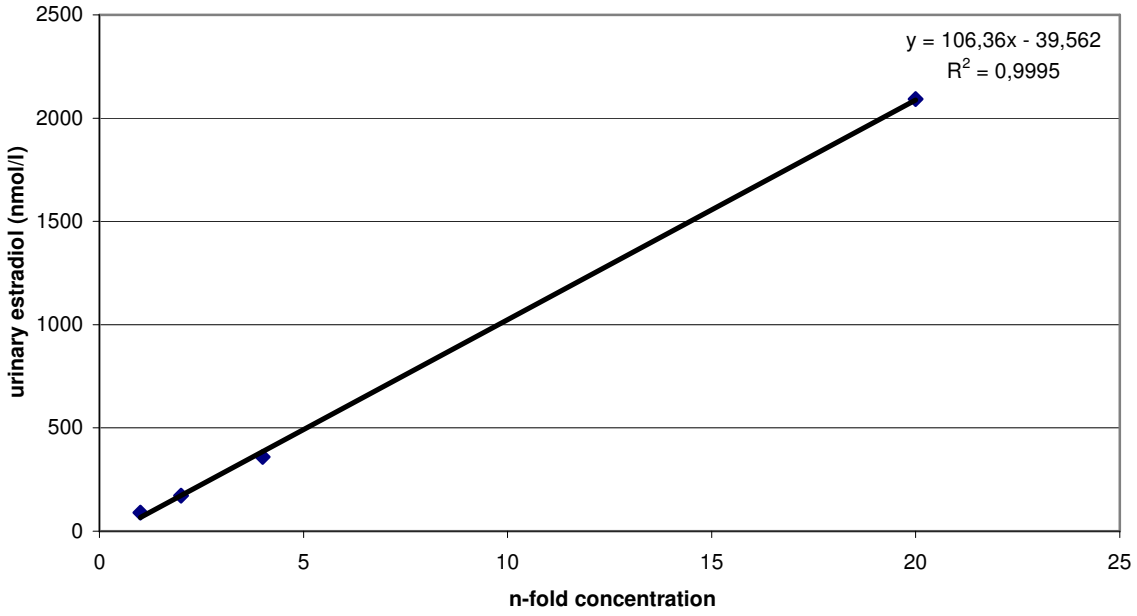


Fig. VII.B9: Regression curve of **estradiol**. Means (three measures per each dilution) of estradiol concentrations show approximate linearity. The dilution series was 1:5, 1:10 and 1:20.

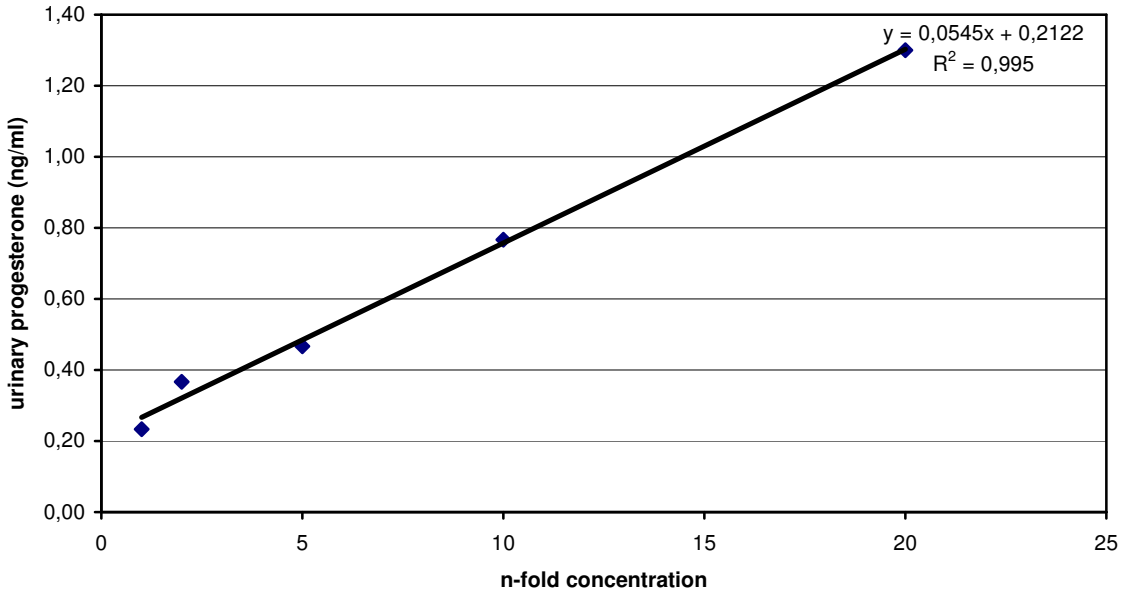


Fig. VII.B10: Regression curve of **progesterone**. Means (three measures per each dilution) of progesterone concentrations showing approximate linearity. The dilution series was 1:5, 1:10, 1:20 and 1:50.

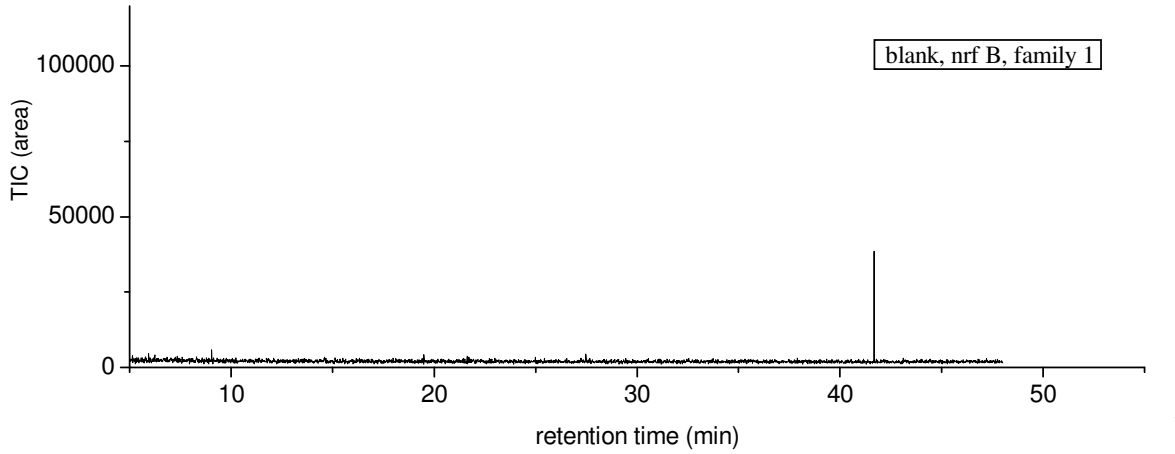


Fig. VII.C1: Blank of **TIC** (total ion chromatogram) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from individual nrf B of family one of an Ansell's *Fukomys* mole-rat (without solvent delay) **illustrating clean fibre** of the SPME-syringe and **clean column** of the gas chromatograph.

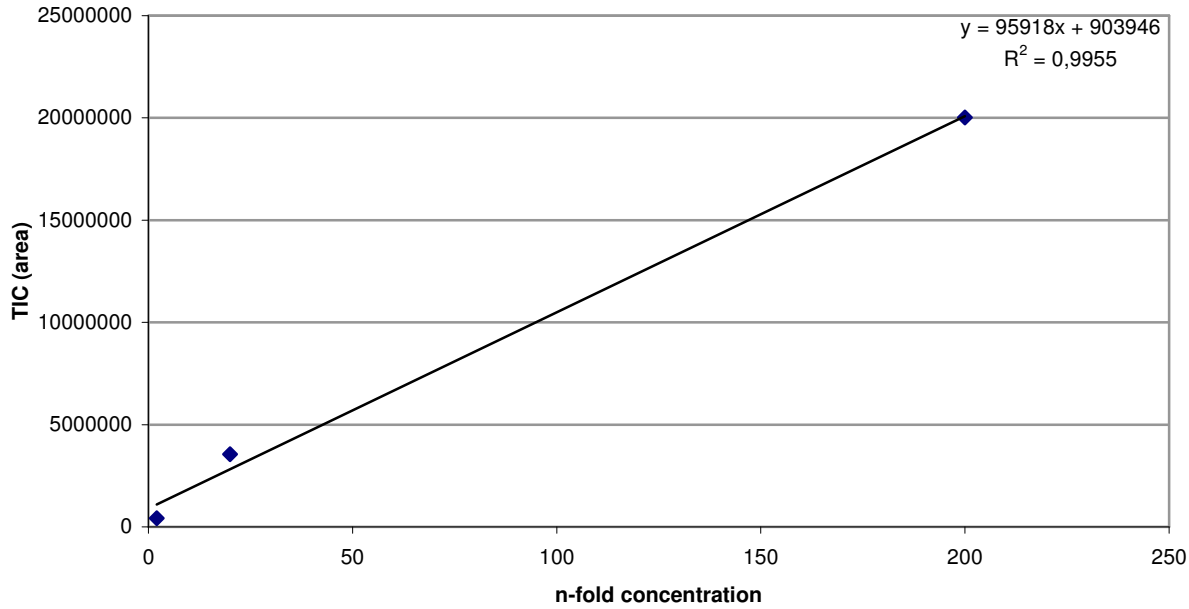


Fig. VII.C2: Regression curve of **vertinpropionate** (three measures per each dilution) by **HS-SPME-GS-MS** (head-space solid-phase microextraction gas chromatography and mass spectrometry). Means of TIC (total ion chromatogram) show approximate linearity. The dilution series was 1: 2,000; 1:20,000 and 1: 200,000.

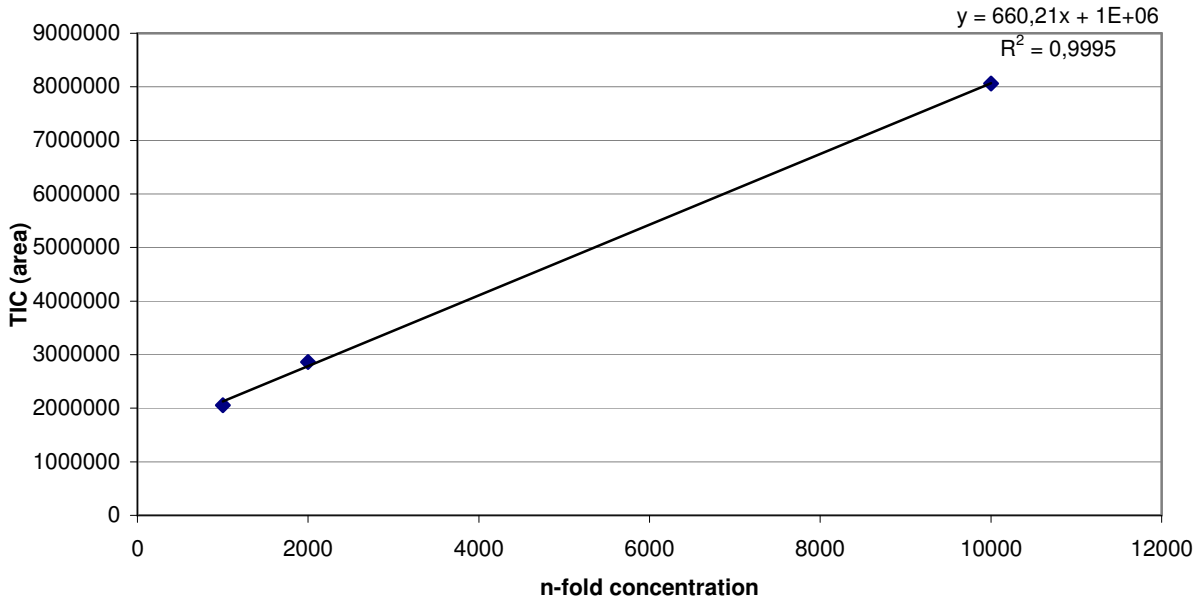


Fig. VII.C3: Regression curve of **1-(S)-α-pinene** (three measures per each dilution) by **HS-SPME-GS-MS** (head-space solid-phase microextraction gas chromatography and mass spectrometry). Means of TIC (total ion chromatogram) show approximate linearity. The dilution series was 1: 1,000; 1: 5,000; 1: 10,000.

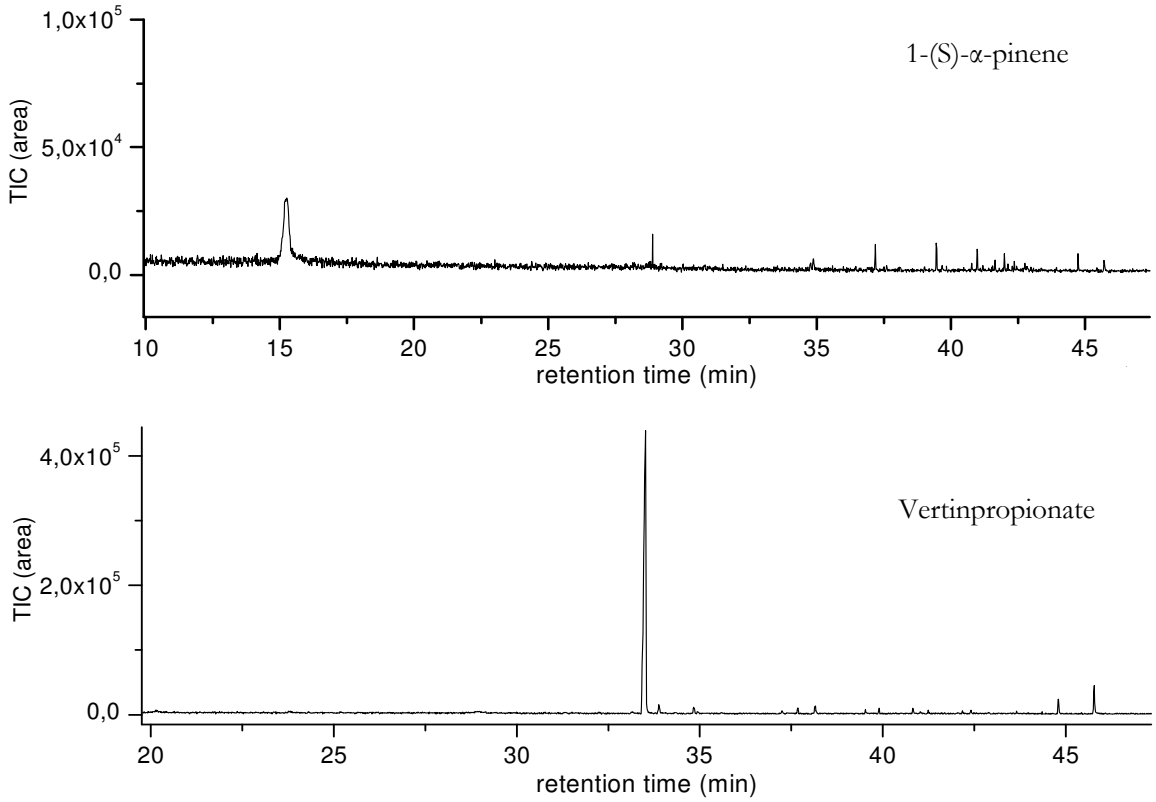
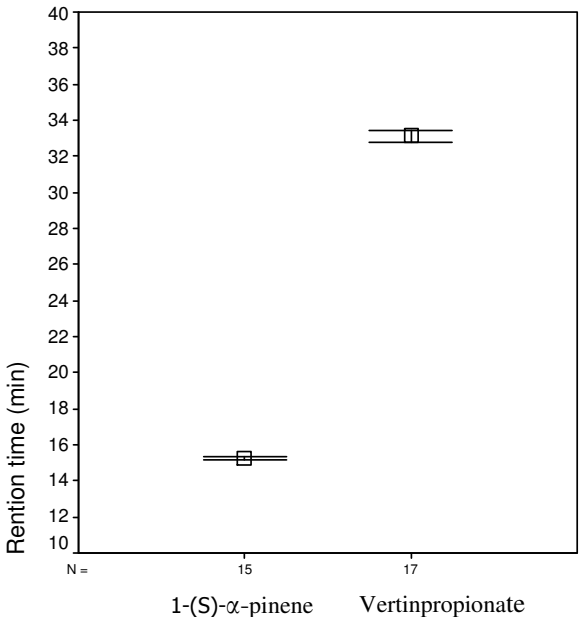


Fig. VII.C4: TIC (total ion chromatogram) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry). Peaks are of 1-(S)-α-pinene and vertinpropionatee in ddH_2O, illustrating appropriateness as calibration compounds.



retention time (min)

STANDARD	mean	N	SD
1-(S)-α -pinene	15,234	15	,1799
Vertinpropionate	33,149	17	,6486
altogether	24,751	32	9,0957

Fig. VII.C5: Validation of the retention time (RT in min) of the **internal standards** 1-(S)-α–pinene and vertinpropionate and their mean values of several GC-MS-measures, illustrating small error bars (with 95% lower and upper confidence intervals, CI; $\alpha = 0.05$) left and standard deviation (SD) right.

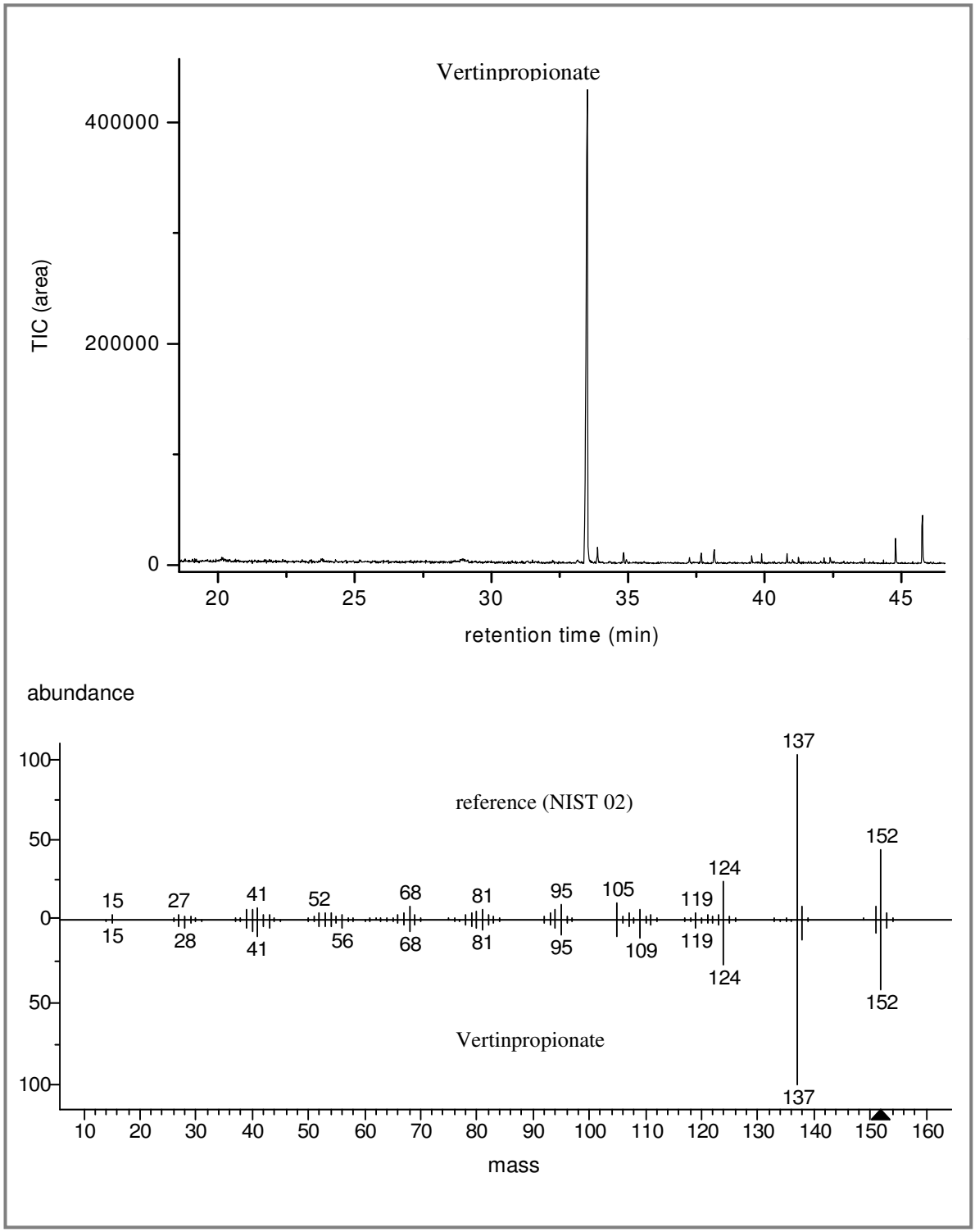


Fig. VII.C6a: TIC (total ion chromatogram) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of dd H_2O-sample. The **mass spectrum** of **vertinpropionate** (2-methoxy-3-(1-methylethyl)-pyrazine) below demonstrates a match of 935 and a probability of 92.5 % (NIST 2002), thus a high similarity of vertinpropionate to its reference (NIST 2002). Dilution of vertinpropionate: 1 µl (1: 2,000 EtOH) in 1,800 µl H_2O (RT = 33.38 min). RT = retention time (min).

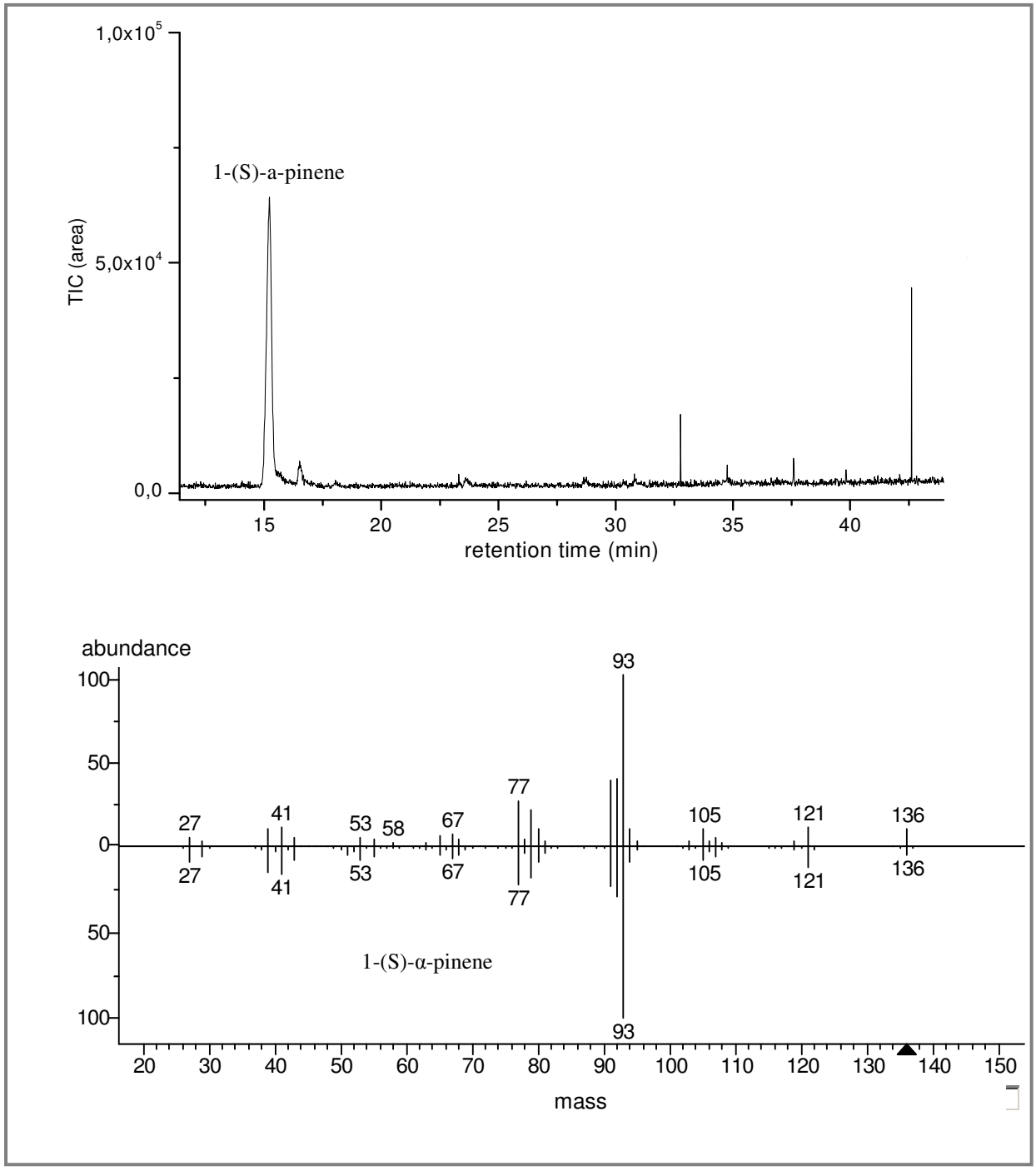


Fig. VII.C6b: TIC (total ion chromatogram) of HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a dd H_2O-sample. The **mass spectrum** of **1-(S)-α-pinene** below demonstrates a match of 918 (NIST 2002), thus a high similarity of 1-(S)-α-pinene to its reference (NIST 2002). Dilution of 1-(S)-α-pinene: 1 µl (1: 1000 ethanol) in 1,800 µl H_2O (RT = 15.20 min). RT = retention time (min).

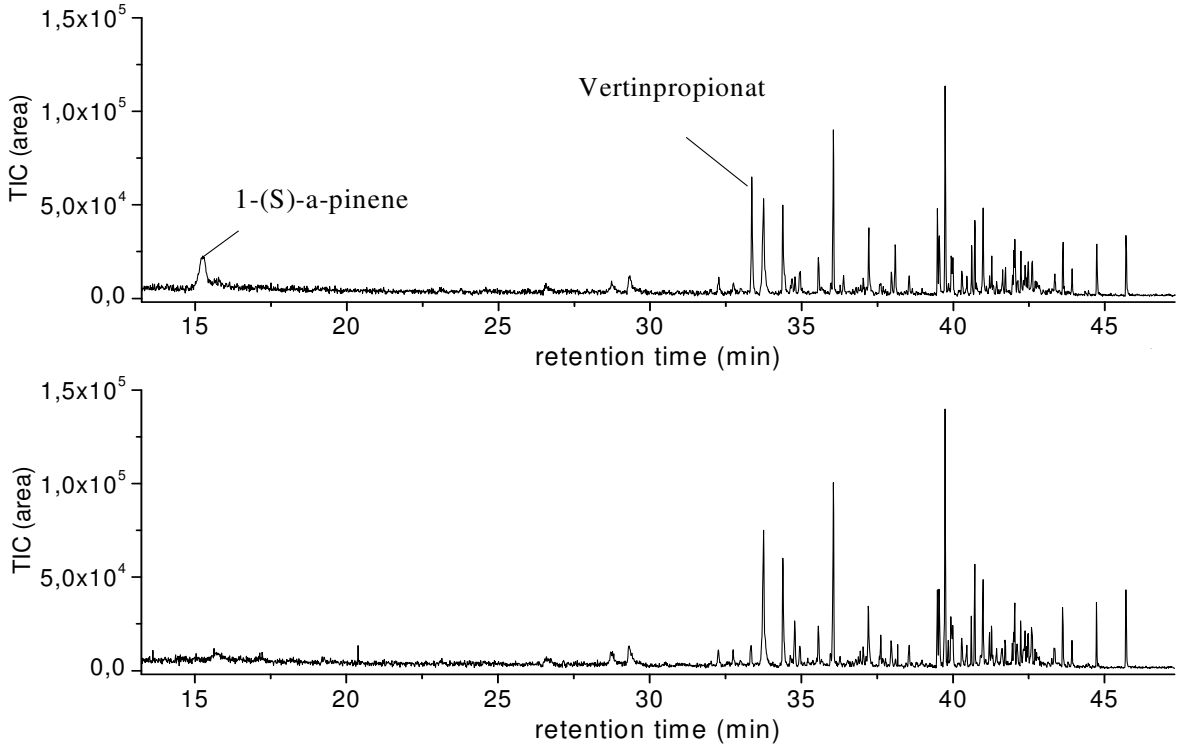


Fig. VII.C7a: TICs (total ion chromatograms) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from family four of hybrid *Fukomys* mole-rats. Peaks are with and without internal standards (IS) **1-(S)-α-pinene** and **vertinpropionate**, illustrating their appropriateness and the masking effect of urinary peak at RT = 33.4 min by vertinpropionate.

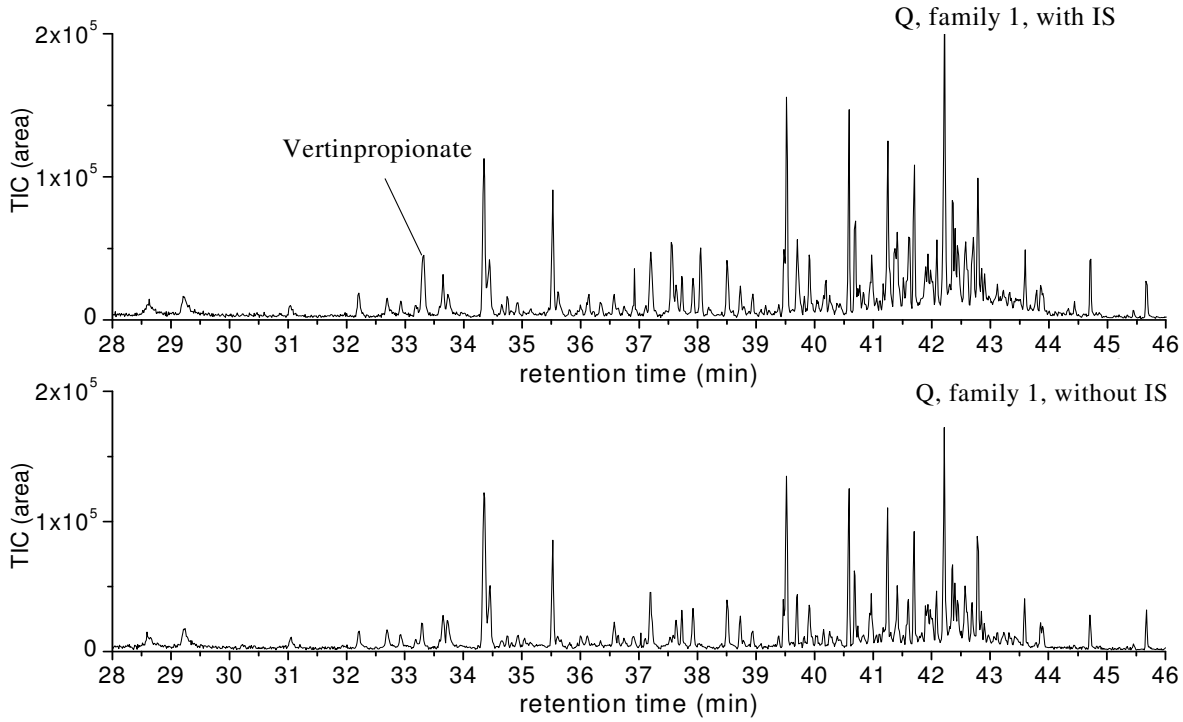


Fig. VII.C7b: TICs (total ion chromatograms) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from the queen (Q) of family one of an Ansell's *Fukomys* mole-rat. Peaks are **with** and **without** the **internal standard (IS) vertinpropionate**, illustrating their appropriateness and the masking effect of urinary peak at RT = 33.4 min by vertinpropionate.

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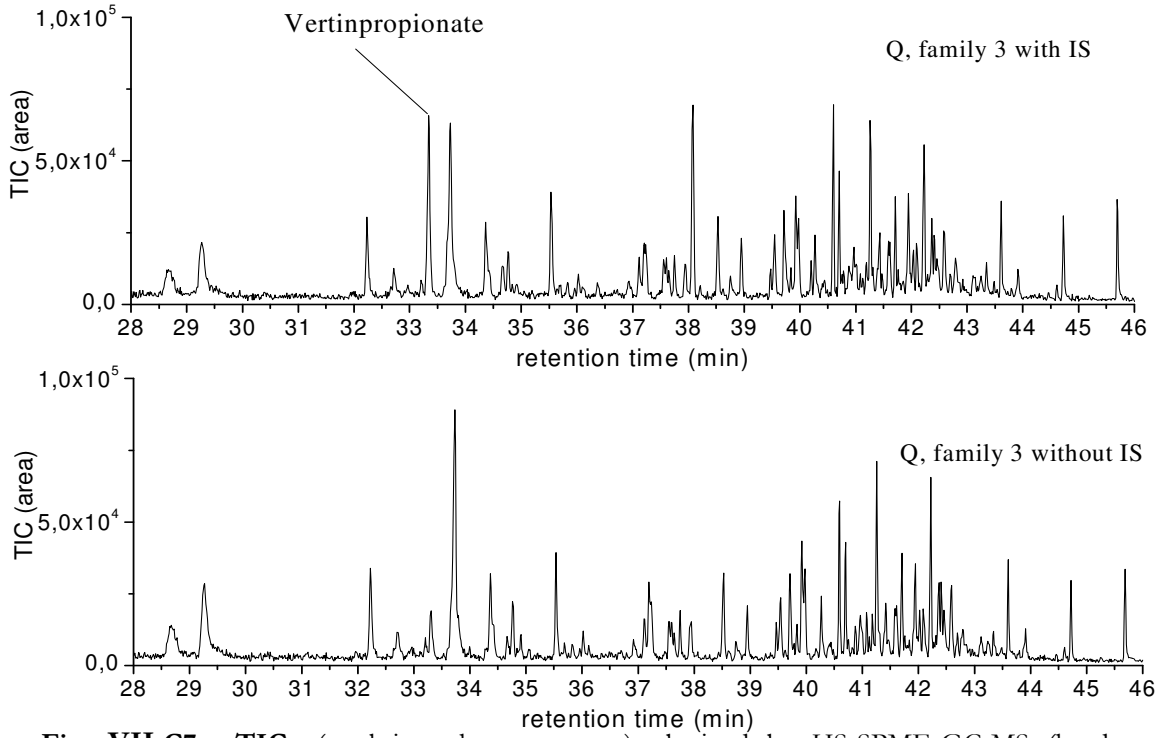

Fig. VII.C7c: TICs (total ion chromatograms) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from the queen (Q) of family three of an Ansell's *Fukomys* mole-rat. Peaks are **with** and **without the internal standard (IS) vertinpropionate**, illustrating their appropriateness and the masking effect of urinary peak at RT = 33.4 min by vertinpropionate.

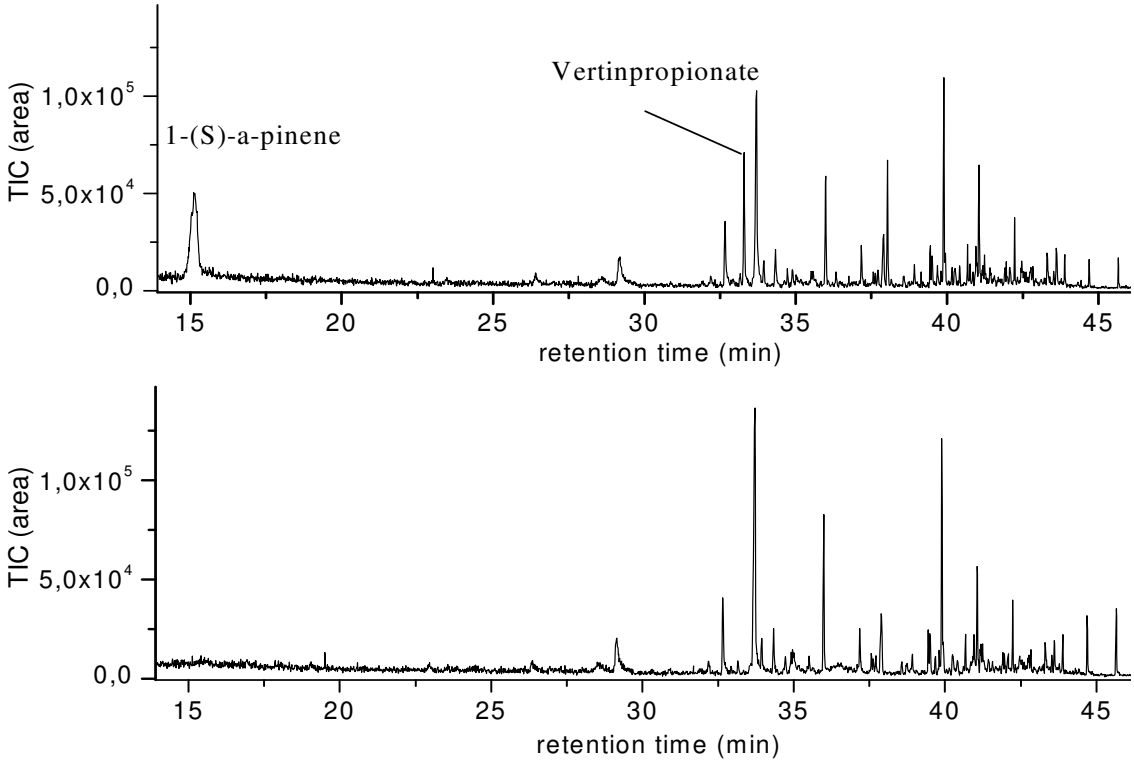

Fig. VII.C8: TICs (total ion chromatograms) of two measures obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from family two of Ansell's *Fukomys* mole-rats during **potato diet**, illustrating **similarity of repeated measures.**

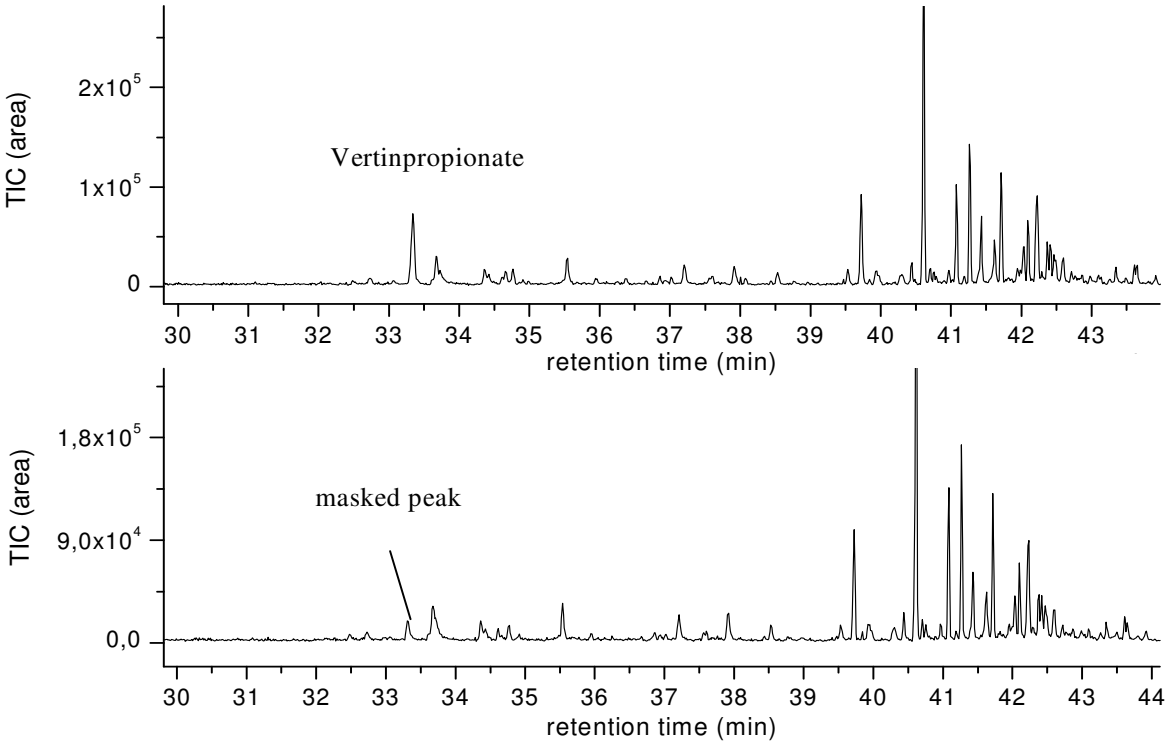

Fig. VII.C9: TICs (total ion chromatograms) of two measures obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a urine sample from family two of Ansell's *Fukomys* mole-rats during **carrot diet**, illustrating **similarity of repeated measures**.

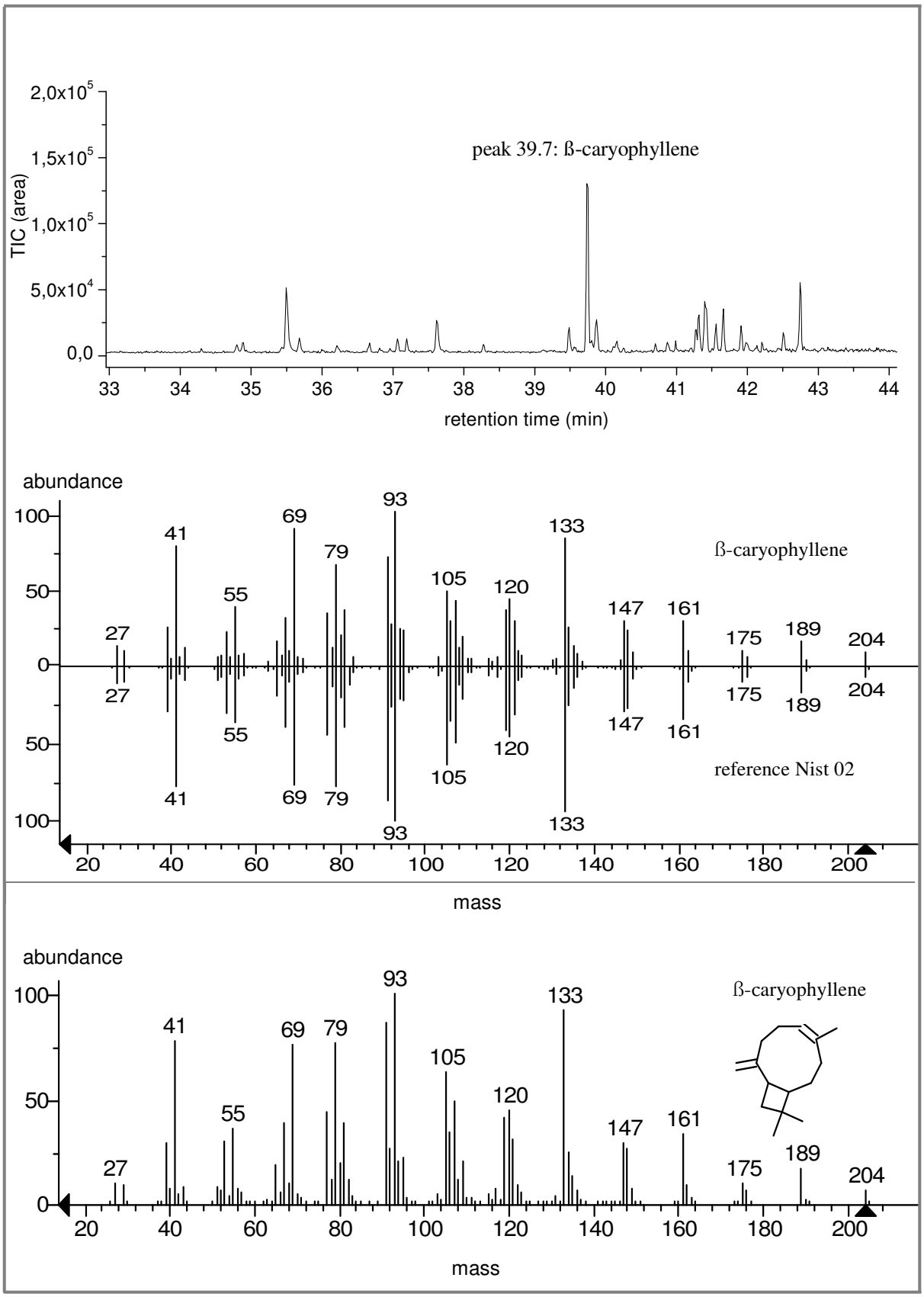

Fig. VII.C10: TIC (total ion chromatogram) obtained by HS-SPME-GC-MS (head-space solid-phase microextraction gas chromatography and mass spectrometry) of a dd H_2O-sample illustrating validation of the retention time (RT in min) of peak 23 (ß-caryophyllene) in the urinary volatiles of mole-rats (extra peaks are dirt of the standard substance). The **mass spectrum** below demonstrates **identification of ß-caryophyllene** with a match of 928 (NIST 2002).

VII.C Table legends

Tab. VII.B1: Mann-Whitney *U*-test of urinary steroid concentrations (estradiol (nmol/mg Cr), progesterone (ng/mg Cr), between different categories of females. SA = sexual active females (rQ = reproductive queens), NSA = not sexual active females (nrQ = non-reproductive queens and nrf = non-reproductive females) during the 1st test phase.

Tab. VII.B2: Friedman's rank test of urinary steroid concentrations (estradiol (nmol/mg Cr), progesterone (ng/mg Cr), between different test-phases.

Tab. VII.B3: Wilcoxon-rank-test of urinary steroid concentrations (estradiol (nmol/mg Cr) of females, progesterone (ng/mg Cr), between different test-phases. + = copulation during the first half hour of test-phase three. - = no copulation during the first half hour of test-phase three.

Tab. VII.B4: Wilcoxon-rank-test of urinary testosterone concentrations (nmol/mg Cr) of males, between different test-periods. nrm = non-reproductive male, nrK non-reproductive king (male nrK1 had already a history of breeding and still lived reproductively inactive but regularly copulating in his family).

Tab. VII.C1: Comparison of SPME equilibration between from different SPME fibres for the head space or immersion transfer from the urinary compounds to the fibre during varying test conditions. The second test condition (in bold) was chosen. x = conducted condition.

Tab. VII.D1: Mean of the total protein concentrations of each species and of all species together. N = number of the urine from the tested individuals. SD = standard deviation.

Tab. VII.D2: Mann-Whitney *U*-test for mean urinary total protein concentrations (mg/mg Cr) between males and females of different genera. p = significance level, n = number of tested urine samples.

Tab. VII.D3: Result of 2D-PAGE of laboratory mouse (strain C57BL/6), *Fukomys* and *Spalax*, broad and short range pI (isoelectric point). Numbers after the specimens refer to numbers of lines in fig. IV.4.3 and fig. IV.4.4 respectively. Q = queen. Colours highlight dots that correlate with one another according to pI and/or mass.

Tab. VII.B1: Mann-Whitney *U*-test of urinary **mean steroid concentrations** (estradiol (nmol/mg Cr), progesterone (ng/mg Cr), between different categories of females. **SA** = sexual active females (rQ = reproductive queens), **NSA** = not sexual active females (nrQ = non-reproductive queens and nrf = non-reproductive females) during the 1st test phase.

test-phase	**paired tests**	**estradiol (nmol/mg Cr)**					**progesterone (ng/mg Cr)**				
		female category	$\bar{x}$ ± SD	n	p	U	**female category**	$\bar{x}$ ± SD	n	p	U
1	**SA/ NSA**	SA	**228059 ± 523660**	88	**0.000**	204.0	SA	**35.1 ± 110.1**	96	**0.001**	1468
		NSA	**4536 ± 5467**	41			NSA	**12.9 ± 17.4**	47		

Tab. VII.B2: Friedman's rank test of urinary steroid concentrations (estradiol (nmol/mg Cr), progesterone (ng/mg Cr), between different test-phases.

test-animal	**estradiol** (nmol/mg Cr)					**progesterone** (ng/mg Cr)				
	χ^2	p	n	df	Wilcoxon	χ^2	p	n	df	Wilcoxon
rQ5	6.0	**0.05**	4	2	+	5.0	0.779	4	2	+
nrQ1	7.6	**0.022**	5	2	+	3.5	0.174	4	2	-
nrQ2	4.6	0.097	3	2	+	4.5	0.104	5	2	-
nrf2	3.7	0.156	4	2	+	8.8	0.012	5	2	**+**
nrf3	2.0	0.368	4	2	-	0.2	0.905	5	2	-
nrf4	2.0	0.360	4	2	+	0.4	0.819	4	2	+
group	**estradiol** (nmol/mg Cr)					**progesterone** (ng/mg Cr)				
	χ^2	p	n	df	Wilcoxon	χ^2	p	n	df	Wilcoxon
all nrf	1.3	0.531	12	2	-	2.9	0.234	14	2	-

Tab. VII.B3: Wilcoxon-rank-test of urinary steroid concentrations (estradiol (nmol/mg Cr) of females, progesterone (ng/mg Cr), between **different test-periods**. + = copulation during the first half hour of test-period three. - = no copulation during the first half hour of test-period three.

category	test-animal	copulation	estradiol						progesterone					
			$\bar{x} \pm SD$ *n*			p paired test			$\bar{x} \pm SD$ *n*			p paired test		
			1	2	3	1-2	2-3	1-3	1	2	3	1-2	2-3	1-3
rQ	rQ5	+	28,407 ± 22,834 *n* = 20	46,779 ± 51,559 *n* = 4	262,059 ± 93,539 *n* = 5	**0.068** Z = -1.826	**0.068** Z = -1.826	**0.043** Z = -2.023	24.3 ± 24.4 *n* =20	34.9 ± 17.9 *n* = 4	66.3 ± 79.1 *n* = 5	0.068 Z = -1.826	0.715 Z = -0.365	**0.043** Z = -2.023
nrQ	nrQ 1	+	2,355 ± 2,623 *n* = 18	2,329 ± 1,754 *n* = 4	89,233 ± 66,255 *n* = 5	0.730 Z = -1.095	**0.068** Z = -1.826	**0.043** Z = -2.023	10.6 ± 16.6 *n* =18	0 ± 0 *n* = 4	4.5 ± 10.1 *n* = 5	0.317 -1.000	0.317 -1.000	0.655 -.447
	nrQ 2	+	5,290 ± 4,970 *n* = 3	11,026 ± 6,700 *n* = 5	8,847 ± 7,970 *n* = 5	0.109 Z = -1.604	0.686 Z = -0.405	1.000 Z = 0.000	28.8 ± 29.0 *n* = 4	2.8 ± 6.8 *n* = 6	14.9 ± 6.8 *n* = 5	0.274 Z = -1.095	0.043 Z = -2.023	0.465 Z = -.730
nrf	nrf2	+	758 ± 1,194 *n* = 19	0.0 ± 0.0 *n* = 6	318 ± 453 *n* = 5	0.109 Z = -1.604	0.180 Z = -1.342	1.000 Z = 0.000	37.3 ± 49.3 *n* =19	2.7 ± 6.5 *n* = 6	2.7 ± 6.1 *n* = 5	**0.043** Z = -2.023	0.655 Z = -0.447	**0.043** Z = -2.023
	nrf3	+ (diff. male)	44 ± 98 *n* = 5	219 ± 536 *n* = 6	103 ± 168 *n* = 6	0.655 Z = -0.447	0.655 Z = -0.447	0.285 Z = -1.069	5.5 ± 12.3 *n* = 5	2.4 ± 5.9 *n* = 6	2.5 ± 3.8 *n* = 6	0.655 Z = -.447	1.000 Z = .000	0.655 Z = -.447
	nrf4	-	8,131 ± 6,719 *n* = 4	18,465 ± 23,477 *n* = 4	24,479 ± 20,495 *n* = 4	1.000 Z = 0.000	0.465 Z = -0.730	1.000 Z = 0.000	4.8 ± 7.3 *n* = 5	39.2 ± 56.6 *n* = 4	0.8 ± 1.6 *n* = 4	0.144 Z = -1.461	0.144 Z = -1.461	0.180 Z = -1.342
all nrf			2434 ± 5283 *n* = 33	4795 ± 11634 *n* = 21	6675 ± 14614 *n* = 15	0.753 Z = -0.314	0.263 Z = -1.120	0.799 Z = -0.255	29.3 ± 42.6 *n* =33	12.9 ± 27.5 *n* =21	2.1 ± 4.1 *n* =15	0.173 Z = -1.363	0.173 Z = -1.362	**0.050** Z = -1.960

Tab. VII.B4: Wilcoxon-rank-test of urinary **testosterone** concentrations (nmol/mg Cr) of males, between different test-periods. nrm = non-reproductive male, nrK non-reproductive king (male nrK1 had already a history of breeding and still lived reproductively inactive but regularly copulating in his family).

category	test-animal	copu-lation	testosterone					
			$\bar{x} \pm$ SD			p paired test		
			1	2	3	1-2	2-3	1-3
nrK	nrK1	+	0.080 ± 0.037 *n* = 5	0.045 ± 0.033 *n* = 6	0.128 ± 0.061 *n* = 6	0.176 Z = -1.355	**0.028** Z = -2.201	0.225 Z = -1.214
nrm	nrm1	+	0.050 ± 0.057 *n* = 2	**0.250** ± 0.199 *n* = 5	0.208 ± 0.175 *n* = 4	0.180 Z = -1.342	0.715 Z = -0.365	0.180 Z =-1.342
	nrm2	+	0.058 ± 0.041 *n* = 5	0.060 ± 0.075 *n* = 6	0.148 ± 0.095 *n* = 6	0.892 Z = -0.135	0.345 Z = -0.943	0.225 Z = -1.214
	nrm3	-	0.060 ± 0.014 *n* = 2	0.130 ± 0.247 *n* = 4	0.056 ± 0.020 *n* = 5	0.655 Z = -0.447	0.713 Z = -0.368	0.157 Z = -1.414
	nrm4	+	0.066 ± 0.059 *n* = 4	0.044 ± 0.045 *n* = 5	0.034 ± 0.056 *n* = 5	0.581 Z = -0.552	0.683 Z = -0.408	**0.066** Z = -1.841
	nrm5	-	0.020 ± 0.007 *n* =5	0.048 ± 0.029 *n* =5	0.023 ± 0.005 *n* =3	**0.039** Z = -2.060	0.564 Z = -0.577	0.317 Z = -1.000
all nrm			0.048 ± 0.039 *n* = 18	0.067 ± 0.113 *n* = 20	0.074 ± 0.078 *n* = 19	0.482 Z = -0.703	0.472 Z = -0.720	0.844 Z = -0.196

Tab. VII.C1: Comparison of **SPME equilibration** between from different SPME fibres for the head space or immersion transfer from the urinary compounds to the fibre during **varying test conditions**. The second test condition (in bold) was chosen. x = conducted condition.

No.	fibre	temp. [° C]	time [min]	immer-sion	head-space	splitless [min]	peak presence
1	DVB/Carboxene/PDMS	110	60		x	2	many peaks
2	**DVB/Carboxene/PDMS**	**80**	**60**		**x**	**2**	**many peaks**
3	DVB/Carboxene/PDMS	50	60		x	2	no peaks
4	DVB/Carboxene/PDMS	50	60		x	2	no peaks
5	DVB/Carboxene/PDMS	50	120	x		2	no peaks
6	Carboxene/PDMS	50	60		x	2	no peaks
7	Carboxene/PDMS	80	60		x	2	no peaks
8	Carboxene/PDMS	110	60		x	2	no peaks
9	Carboxene/PDMS	50	60	x		2	no peaks
10	PDMS	110	60		x	2	no peaks
11	PDMS	50	60	x		2	no peaks
12	PDMS	100	60		x	2	no peaks
13	PDMS	80	60		x	2	no peaks

Tab. VII.D1: Mean of the total protein concentrations of **each species** and of all species together. N = number of the urine from the tested individuals. SD = standard deviation.

SPECIES	mean	N	SD
F. anselli	0.61	10	1,03
F. kafuensis	0.53	10	0,77
hybrides	0.65	13	1,98
Spalax	0.25	22	0,53
Spalacopus	0.37	13	0,81
altogether	0.44	68	1,08

Tab. VII.D2: Mann-Whitney *U*-test for mean urinary **total protein concentrations** (mg/mg Cr) between males and females of different genera. p = significance level, n = number of tested urine samples, Cr = creatinine

	Fukomys				***Spalax***				***Spalacopus***				**C57BL/6**			
sex	mean $\bar{x}$ ± SD	n	p	U	mean $\bar{x}$ ± SD	n	p	U	mean $\bar{x}$ ± SD	n	p	U	mean $\bar{x}$ ± SD	n	p	U
♂	0.3 ± 0.4	17	0.90	132.0	0.3 ± 0.6	16	0.23	31.0	0.6 ± 1.0	8	0.37	10.0	9.5 ± 3.5	10	**0.01**	7.0
♀	1.0 ± 1.9	16			0.0 ± 0.0	6			0.0 ± 0.0	4			15.7 ± 2.5	9		

Tab. VII.D3: Result of **2D-PAGE** of laboratory mouse (strain C57BL/6), *Fukomys* and *Spalax*, broad and short range pI (isoelectric point). Numbers after the specimens refer to numbers of lines in fig. IV.4.3 and fig. IV.4.4 respectively. Q = queen. Colours highlight dots that correlate with one another according to pI and/or mass.

	pI = 3-10			**pI = 3.9-5.1**		
Species	**dot**	**mass**	**pI**	**dot**	**mass**	**pI**
laborarory mouse	1	~19 kDa	4.4-4-9	1	~19.2 kDa	4.6
	2	~19.5 Da	5.0-5.4	2	~19.2 kDa	4.6-4.7
				3	~19 kDa	4.7
				4	~19 kDa	4.8
F. kafuensis x *anselli* #11 ♂	1	20 kDa	4.5-4.7	1	~20 kDa	4.1
	2	~22 kDa	5.0-5.4	2	~20 kDa	4.1-4.2
	3	~14 kDa	5.4-5.6	3	~20 kDa	4.3-4.4
				4	~22 kDa	4.5
				5	~22 kDa	4.6
				6	~22.5 kDa	4.6
F. kafuensis x *anselli* #14 ♂				1	~21 kDa	4.0
				2	~21 kDa	4.1
				3	~23 kDa	4.4-4.5
F. kafuensis x *anselli* #4 Q				1	~20 kDa	4.0-4.1
				2	~23 kDa	4.4
				3	~23 kDa	4.5
F. anselli #19 ♂				1	~20 kDa	4.1
Spalax carmeli #5 ♂	1	~11 kDa	6.6-6.7			
	2	~14 kDa	6.7-6.9			
Spalax carmeli #7 ♂	1	~14 kDa	4.5-4.7			
	2	~16 kDa	4.6-5.0			
	3	20 kDa	5.1-5.3			
	4	~19 kDa	5.6-5.7			
	5	~19 kDa	5.8-6.0			
	6	~18,5 kDa	6.1-6.2			
	7	~11-12 kDa	6.7-6.8			

VII.D Acknowledgement

I feel deeply grateful to my doctoral advisor Professor Dr. Hynek Burda, who encouraged and supported me to work on this PhD-thesis in his group, with a topic which has fascinated me since my early master study. Always, he was open-minded and inspiring in scores of discussions to exceptionally appearing ideas. During my stays in Prague he gave me free accommodation, making me feel at home, and optimistically inspired me through phases of uncertainties.

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After many endless, inspiring talks, my husband Olaf Hagemeyer nearly became a "pheromone-expert". Thank you, Olaf, for this, the mental support and for long years understanding for my steadily being busy and occupied in the lab.

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VII.E Curriculum vitae

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SCHULISCHE AKTIVITÄTEN:

seit 10/2009	Vorsitz der Fachschaft Biologie
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03/2000	Ethologie-Praktikum am Department of Zoology, University of Oxford

KONFERENZEN/TAGUNGEN/KURSE:

08/2008	CENTRAL EUROPEAN MEETING ON MOUSE EPIGENETICS, Nove Hrady, Tschechien Vortrag "The influence of chemical stimulus and sexual activity on eusocial mole-rats (genus: *Fukomys*)"
10/2005	EINLADUNG ZUM VORTRAG IM ZENTRALLABOR DER MEDIZINISCHEN UNIVERSITÄTSKLINIK, Essen, Deutschland. Vortrag „Das ungewöhnliche Leben der sambischen Graumulle (Gattung: *Cryptomys*)"
09/2005	79 JAHRESTAGUNG DER DEUTSCHEN SÄUGETIERKUNDLICHEN GESELLSCHAFT, Essen, Deutschland. Poster "The sexual status of female Zambian mole-rats (*Cryptomys* sp., Bathyergidae) affects their smell attractiveness"
11/2004	CHEMICAL ANALYSIS IN CHEMICAL ECOLOGY AND ECOTOXICOLOGY, POSTGRADUATE COURSE, Lund University, Sweden. Vortrag "Different aspects and difficulties regarding the analytical chemistry of mole-rats´ odour sources"
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07/2004	POST-CONFERENCE OLFACTION FIELD WORKSHOP, Kampinos Nationalpark, Catholic University of Lublin, Poland
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WS 2005	Einführung in die Zoologie: Sinneswahrnehmung (Chemoperzeption und Photoperzeption)
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VII.F List of Publications

HAGEMEYER P, LANGE S, BROECKER-PREUSS M & BURDA H (2009) The influence of olfactory stimulus and sexual activity on gonadal steroids in eusocial mole-rats. Folia Zoologica 58: 65-74.

HAGEMEYER P (2007) Durchführung und Reflexion eines bilingualen Moduls „Duftstoffe" im Differenzierungskurs Chemie (Biologie) der Jahrgangstufe 10 zur Heranführung der Schüler an die Wissenschaftssprache Englisch. Examensarbeit, Studienseminar Essen.

HAGEMEYER P & BEGALL S (2006) Individual odour similarity and discrimination in the coruro (*Spalacopus cyanus*, Octodontidae), Ethology 112: 529-536.

LANGE S, NEUMANN B, **HAGEMEYER P** & BURDA H (2005) The smell of carrots: Kairomone guided food location in subterranean mole-rats, *Cryptomys* sp. (Bathyergidae). Folia Zoologica 54: 263-268.

HAGEMEYER P (2002) Genetic polymorphism and biological function of MUP (major urinary proteins) in the house mouse. Examensarbeit, Universität Duisburg-Essen.

Abstracts:

HAGEMEYER P, LANGE S & BURDA H (2005) The sexual status of female Zambian mole-rats (*Cryptomys* sp., Bathyergidae) affects their smell attractiveness. *Deutsche Jahrestagung der Gesellschaft für Säugetierkunde, Essen* (Germany), 18.-2.9.2005. Mammalian Biology 70s: pp 16-17.

HAGEMEYER P, PAPENFUHS N, PEUCKMANN S & BURDA H (2004) Chemical communication in blind, subterranean eusocial mole-rats (genus *Coetomys*): Which odour source codes information for individual recognition? *5th International Symposium on Physiology, Behaviour and Conservation of Wildlife, Berlin* (Germany), 26.-29.9.2004. Advances in Ethology 38s: p 56.

HAGEMEYER P, Papenfuhs N, Peuckmann S & BURDA H (2004) Individual recognition via chemical communication as an incest avoidance mechanism in Zambian mole-rats (*Coetomys*)? *9th International Conference "Rodents at Spatium", Lublin* (Poland), 12.-14.7.2004: p 37.

VII.G Assertion

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Math.-Nat.- Fachbereiche zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema *„Pheromones in social mole-rats and implications for the study the mammalian chemical communication"* zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von (Petra agemeyer) befürworte.

Essen, den _____ _____

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 6 der Promotionsordnung der Math.-Nat.- Fachbereiche zur Erlangung der Dr. rer. nat., dass ich die vorliegende Disseration selbstständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, den _____ _____

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 8 der Promotionsordnung der Math.-Nat.- Fachbereiche zur Erlangung der Dr. rer. nat., dass ich keine anderen Promotionen oder Promotionsversuche in der Vergangenheit durchgeführt habe und das diese Arbeit von keiner anderen Fakultät abgelehnt worden ist.

Essen, den _____ _____