Chapter 6
The Role of Bacteria in Chemical Signals of Elephant Musth: Proximate Causes and Biochemical Pathways

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6.1 Introduction

6.1.1 Chemical Signals of Elephant Musth

Mammalian males of many species have long been noted for their use of secretions and excretions for conspecific communication. In 1871, Charles Darwin spoke to this topic: "The males are almost always the wooers; and they alone are

This chapter is dedicated to Dr. Eric S. Albone for his pioneering research on the role of bacteria in mammalian semiochemistry.

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armed with special weapons for fighting with their rivals. They are provided, either exclusively or in a much higher degree than the females, with odoriferous glands. There is another and more peaceful kind of contest, in which the males endeavour to excite or allure the females by various charms. This may be affected by the powerful odours emitted by the males during the breeding-season; the odoriferous glands having been acquired through sexual selection" (Darwin 1981, p 313, 397; Kappeler 1998).

Darwin's characterizations are certainly relevant for male African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephants in musth, a periodic state which is typically characterized by elevated serum androgens (specifically testosterone and dihydrotestosterone), heavy drainage from the temporal glands, urine dribbling, appetite reduction, increased activity to search for mates, intense competition for mating, and increased aggression (Eisenberg et al. 1971; Jainudeen et al. 1972; Poole and Moss 1981; Poole et al. 1984; Hall-Martin and van der Walt 1984; Poole 1987; Poole 1989; Rasmussen et al. 1996; Schulte and Rasmussen 1999; Ganswindt et al. 2005a, b; Brown et al. 2007; Rasmussen et al. 2008; Chelliah and Sukumar 2013; Ghosal et al. 2013). Unlike the rut in ungulates (e.g., moose and deer), musth among male Asian and African elephants in any given population is asynchronous.

African and Asian elephants have well-developed primary and secondary (vomeronasal) olfactory systems that are used to detect Darwin's "powerful odours" (chemical signals) from conspecifics (Rasmussen and Schulte 1998; Rasmussen et al. 2003). The number of confirmed mammalian chemical signals is relatively small (Albone 1984; Brown and Macdonald 1985; Schildknecht and Ubl 1986; Brennan and Keverne 2004; Burger 2005; Brennan and Zufall 2006; Sorensen and Hoye 2010; Charpentier et al. 2012; Apps 2013; Liberles 2014; Wyatt 2014). Therefore, it is rather startling that two insect pheromones have been identified as semiochemicals in Asian elephants: (1) *Z*-7-dodecen-1-yl acetate, a urinary signal of impending ovulation (Rasmussen et al. 1997), and (2) frontalin, a chemical signal of sexual state and social maturity in the temporal gland secretion (TGS) of mature males in musth (Rasmussen and Greenwood 2003; Greenwood et al. 2005).

Musth male TGS and urine emit distinctive odors which are easily detected from a great distance by conspecifics as well as humans (Poole 1987; Poole 1989; Hollister-Smith et al. 2008). Darwin (1981, p 279) described the odor of musth TGS as follows: "At this period the glands on the side of the face of the male elephant enlarge and emit a secretion having a strong musky odor." The pungent musth secretions and excretions, which are primarily TGS and urine, serve as vehicles for semiochemicals produced by physiological changes (Rasmussen and Perrin 1999; Schulte and Rasmussen 1999; Ganswindt et al. 2005a, b).

Urine is the solution by which mammals excrete not only excess water and electrolytes but also a myriad of metabolites, some of which function in chemical signaling (Albone 1984) and others in medical diagnosis (Ryan et al. 2011). Albone (1984, p 165) summarized this metabolic messaging as follows: "… urine necessarily conveys to the external world in its detailed composition much information concerning the internal physiological state of the animal concerned and thus provides the necessary basis for the evolution of specialized semiochemical systems."

Sexually dimorphic odors are significant factors in mammalian sexual selection (Blaustein 1981). Accordingly, African elephant musth urine trails evoke strong inspection by conspecifics (Poole 1987). It has been demonstrated that male African elephants can differentiate between the urine of female conspecifics in the luteal and follicular phases of estrus, as well as between musth and non-musth urine (Bagley et al. 2006; Hollister-Smith et al. 2008). The bark beetle pheromones frontalin and *endo*- and *exo*-brevicomin as well as their biosynthetic precursors (alken-2-ones; Vanderwel and Oehlschlager 1992; Keeling et al. 2013) have been identified in the urine of female African elephants, but their putative role in elephant chemical signaling has not yet been verified (Goodwin et al. 2006).

Rasmussen and Wittemyer (2002) reported much higher concentrations of volatile alkan-2-ones in the urine of wild African male elephants in musth when compared to non-musth urine. As this distinctive increase of alkan-2-ones has been demonstrated for both wild and captive male African and Asian elephant excretions and secretions, these authors proclaimed it as a "species-free" chemical signal of musth (Rasmussen and Wittemyer 2002).

Goodwin and colleagues used automated solid phase dynamic extraction (SPDE) and gas chromatography-mass spectrometry (GC-MS) (Goodwin et al. 2007, 2012; Goodwin and Schulte 2009) to find that the abundance of alkan-2-ones, alkan-2-ols, and several simple aromatic compounds is elevated in the headspace over musth urine of captive elephants. The production of these compounds continues in the urine exogenously due to bacterial fermentation. Several studies have shown that chemical changes occur in urine as it ages (Wellington et al. 1983; Saude and Sykes 2007; Mochalski et al. 2012; Kwak et al. 2013; Troccaz et al. 2013). Thus, we proposed that these simple compounds may serve as temporal signals from musth males to conspecifics.

The primary focus of the present report is on the alkan-2-ones and their corresponding alkan-2-ols. Specifically we discuss (1) the probable biosynthesis of these compounds via a secondary pathway for fatty acid metabolism, (2) the proximate cause for their increased abundance in musth urine, and (3) the role of bacteria in the increased abundance of these compounds exogenously in aged urine.

6.1.2 Microorganisms in Mammalian Semiochemistry

In his seminal book on mammalian semiochemistry, Albone (1984) includes a chapter entitled "Microorganisms in Mammalian Semiochemistry." In a subsection of this chapter (p 147–148), Albone discusses a "fermentation hypothesis" of chemical recognition which arose from his research with the red fox (*Vulpes vulpes*; Albone et al. 1974, 1977, 1978; Albone and Perry 1976), as well as from Gorman's research on the Indian mongoose (*Herpestes auropunctatus*; 1974; 1976). These studies propose that microorganisms associated with the anal sac/anal pocket metabolize organic substrates to produce a set of carboxylic acids that function for individual or

group recognition. Simply put, the two tenets of the fermentation hypothesis can be expressed as follows.

- Symbiotic microorganisms in scent glands contribute to mammalian odor profiles
- Variations in mammalian host odor profiles mirror variations in their bacterial communities, and these odors have signaling relevance

The first of these tenets has long been known and amply demonstrated (cf. Leyden et al. 1981; Zechman et al. 1984; Voigt et al. 2005; Natsch et al. 2006; Penn et al. 2007; James et al. 2013), but in some cases it remains to be determined whether the specific metabolites that bacteria contribute are relevant in the context of signaling (Ezenwa and Williams 2014). The second tenet has only recently begun to be demonstrated (Zomer et al. 2009; Sin et al. 2012; Theis et al. 2013; Leclaire et al. 2014).

While the original fermentation hypothesis focused on mammalian scent glands, a broader definition has been proposed: "Although the fermentation hypothesis was developed for mammals that mark with scent glands, it might also accommodate mammals that mark with urine or faeces, or even nonmammalian species that use chemical communication. Therefore viewed broadly, the fermentation hypothesis has the potential to be an inclusive model for animal chemical recognition" (Archie and Theis 2011, p 428).

We propose herein that the fermentation hypothesis is relevant for exogenous, microbially mediated urinary chemical signals of elephant musth. There are many definitions of fermentation in biochemistry and microbiology (Li 2004; Willey et al. 2008); however, for the current context this simple one seems most appropriate: "… a process in which microorganisms produce chemical changes in organic substrates through the action of enzymes produced by these microorganisms (Li 2004, p 685)."

6.1.3 Hypotheses and Predictions

Our experiments were crafted with the following hypotheses in mind: (1) bacterial metabolism is responsible for the exogenous increase in abundance of the selected urinary compounds; (2) a higher abundance of the metabolites of interest would be observed in aged musth urine compared to aged non-musth urine; (3) the abundance of these metabolites could be reduced by centrifuging and filtering the urine to remove bacteria prior to aging; (4) increased bacterial metabolism would be restored by re-introduction of the centrifugation pellet or feces; (5) the bacteria would be killed by autoclaving the centrifugation pellet before reintroduction, thus lowering the abundance of the metabolites of interest; (6) the alkan-2-ones and alkan-2-ols are produced by fatty acid metabolism; therefore, (7) fatty acids would be found in the elephant urine, likely carried there bound to albumin (Kamijo et al. 2002; van der Vusse 2009).

6.2 Materials and Methods

6.2.1 Urine Collection

The elephants used in this research were examined routinely by veterinarians and were in good health at the times of urine collection (for more information about these elephants, see Goodwin et al. 2012, Table 6.1). Musth status was characterized by urine dribbling, heavy temporal gland secretion, and a pungent odor from excretions and secretions. A musth and non-musth urine sample from three African elephants and one Asian elephant were collected in clean, new glass containers by staff members at various facilities (see Acknowledgments), frozen soon after collection on dry ice or in a −70 or −80 °C freezer, and shipped on dry ice to Hendrix College. Samples were stored in a −70 °C freezer and thawed at approximately 37 °C immediately before analysis by SPDE/GC-MS). Bacterial contamination in human urine can alter the metabolic profile over time unless the urine is stored in an ultralow-temperature freezer (Saude and Sykes 2007).

6.2.2 SPDE/GC-MS Sample Preparation and Analysis

SPDE/GC-MS has been previously employed in a study of putative chemical signals in African elephant urine (Goodwin et al. 2006, 2007, 2012). SPDE features concentration of volatile organic chemicals by repetitive flow back and forth over an adsorbant polymer coating on the inside wall of a stainless steel syringe needle that is attached to a gastight syringe (Lipinski 2001). For a typical SPDE/GC-MS analysis of elephant urine, a 1.0 mL aliquot and a small Teflon®-coated stir bar were sealed in a 20 mL glass screw-top vial (autosamplerguys.com), which had been previously washed thrice with acetone and thrice with deionized water and was sterilized by autoclaving at 18 psi and 121 °C for 20 min.

The vials were sealed with a threaded, metallic septum cap (silicone/PTFE layered septum; autosamplerguys.com). Multiple samples were programmed to run automatically using the Combi PAL robot and associated SPDE hardware and software (Chromtech.com). The SPDE needle was internally coated with activated charcoal (Carboxen®)-polydimethylsiloxane (AC-PDMS). After incubating the stirred sample at 37 °C for 15 min, the headspace was extracted for 13 min (200 up-and-down 1 mL strokes of the syringe). Desorption of adsorbed analytes was at 250 °C in the GC inlet. GC-MS analyses were conducted using an Agilent 6890 N GC and 5973 N Mass Selective Detector. The capillary GC column was an Equity 1 (bonded; polydimethylsiloxane), 60 m × 0.32 mm ID, 1 μm film thickness (Supelco cat. No. 28058U). The GC oven was temperature programmed to hold for 2 min at 35 °C, followed by ramping to 180 °C at 3.75 °C/min where it was held for 5 min before ramping at 20 °C/min to a final temperature of 250 °C where it was held for 10 min. The mass spectrometer was programmed at 3.09 scans/s for a mass scan of 30–500 amu. The identities of all compounds reported herein from SPDE/GC-MS were verified by GC retention times and mass spectra when compared to commercial samples, as well as by comparison to the NIST mass spectral library.

6.2.3 Experimental Procedures to Assess Microbial Metabolism

To generate the data shown in Table 6.1, samples of musth and non-musth urine from three healthy African elephants were centrifuged (10–15,000 × *g* for 2 min) and filtered (0.22 μm sterilizing filter) to remove insolubles, including microbes. Glassware, plasticware, and stainless steel spatulas were washed with acetone and deionized water, and sterilized by autoclaving at 18 psi and 121 °C for 20 min. 1 mL aliquots of these centrifuged and filtered samples in SPDE vials were treated as follows in the bulleted list, sealed with a septum cap, and allowed to incubate at room temperature for 48 h before analyzing by SPDE/GC-MS. The abbreviations in parentheses refer to entries in Tables 6.1 and 6.2.

- Nothing was added to centrifuged and filtered musth and non-musth urine (CF)
- A non-musth centrifugation pellet was added to centrifuged and filtered musth and non-musth urine (CFNMP)
- A musth centrifugation pellet was added to centrifuged and filtered musth and non-musth urine (CFMP)
- An autoclaved musth centrifugation pellet was added to centrifuged and filtered musth urine (CFA)
- A stainless steel spatula was inserted into fresh, male African elephant feces; the visible feces was gently wiped off the spatula with a Kimwipe®; then the spatula tip was inserted into centrifuged and filtered musth urine and moved around for a few seconds to introduce fecal bacteria (CFF)

6.2.4 Culturing Bacteria from Elephant Urine

Bacteria were cultured by evenly spreading 0.1 mL of thawed urine over a tryptic soy agar plate using a sterile bent glass rod. The plate was then incubated 48 h at 37 °C. Sequential, selective streaking and culturing led to the isolation of several pure strains of bacteria.

6.2.5 Identifying Bacteria from Elephant Urine

Bacterial identification was based on sequencing the highly conserved 16S rRNA gene. Single colonies were placed in PCR tubes, and amplified using GoTaq(R) Hot Start PCR (Promega Corp., Madison, WI) using universal bacterial primers 8 F and 1492R, resulting in a ca. 1500 bp fragment. This was visualized by agarose gel electrophoresis, and purified with QIAquick PCR purification kits (Qiagen, Alameda, CA). The purified product was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA) and adjusted to suggested concentrations. Samples were sequenced at the University of Arkansas for Medical Sciences (UAMS) Sequencing Core Facility using a 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA), using primers Fd1, 800 F, 1050 F, 800R, 1050R and

RP2. Sequences were aligned and edited using ChromasPro (Technelysium Pty Ltd, Brisbane). Identification was performed using searches for the closest matches in the NIH BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

6.2.6 Urinary Fatty Acids

Concentrations and identities of urinary fatty acids were determined by GC-MS as described by Shoemaker and Elliot (1991).

6.2.7 Sequencing African Elephant Urinary Proteins

Proteins were resolved by SDS-PAGE, visualized by colloidal Coomassie staining, excised, and treated in-gel with trypsin for protein identification (Smart et al. 2009). Peptides were subjected to tandem mass spectrometric analysis by nanospray ionization with a coupled nanoLC 2D reverse-phase liquid chromatography system (Eksigent) and Thermo LTQ mass spectrometer (Smart et al. 2009). Two proteins were identified by UniProt database searching with Mascot using the *Loxodonta africana* protein sequences (http://www.uniprot.org); The UniProt Consortium (2014) Activities at the Universal Protein Resource (UniProt). *Nucl Acids Res* 42(D1): D191–D198). A Schiff stain and a subsequent Coomassie blue stain were used to verify that one of the two proteins is highly glycosylated (Carlsson 1993).

6.2.8 IACUC Approval

All animal-related activities and research discussed herein were approved by the Institutional Animal Care and Use Committee (IACUC) or its equivalent at all participating institutions.

6.3 Results

6.3.1 General Overview

A series of alkan-2-ones, alkan-2-ols, and a few aromatic compounds have a higher concentration in the urine of musth male elephants than in urine from non-musth males (Rasmussen and Wittemyer 2002; Goodwin et al. 2012). After 48 h at the ambient temperature the abundance of these compounds in the expelled urine had increased, and this increase could be prevented by centrifuging and filtering to remove insolubles, including bacteria (Goodwin et al. 2012). In this section we compare relative abundances of these organic compounds in non-musth and musth African male elephant urine that has been aged for 48 h, centrifuged and filtered, and then a musth

or non-musth centrifugation pellet or feces has been reintroduced. We also demonstrate that elephant urine contains fatty acids, the probable biochemical precursor of the alkan-2-ones and alkan-2-ols. Additionally, African elephant albumin, the probable carrier protein that ferries fatty acids into the urine, has been sequenced and shown to share a high sequence homology with Asian elephant albumin.

6.3.2 *Relative Abundance of Selected Compounds in Aged African Elephant Urine*

Following up on earlier findings that microorganisms are likely responsible for the exogenous increase in abundance of certain urinary ketones, alcohols, and aromatic compounds (Goodwin et al. 2012), a new set of experiments were designed to provide additional information regarding this phenomenon.

Tables 6.1 and 6.2 show compound abundances for aged centrifuged and filtered non-musth urine and musth urine (CF), and for centrifuged and filtered non-musth or musth urine with a non-musth or musth centrifugation pellet (CFNMP or CFMP, respectively) reintroduced before aging, as well as for centrifuged and filtered musth

Table 6.1 Comparison of selected compounds in non-musth and musth urine of three male African elephants under various conditions. The data are derived from mean peak areas from GC-MS total ion chromatograms normalized to the value for non-musth CF pentan-2-one, the compound of lowest abundance[a]

	Non-musth urine			Musth urine				
Compound	CF	CFNMP	CFMP	CF	CFA	CFF	CFNMP	CFMP
Pentan-2-one	1.00[b]	1.81[b]	3.96[b]	82.3	91.6[b]	**167**[b]	116	115
Hexan-2-one	–	–	1.63[b]	57.0	48.3[c]	92.7[c]	79.9	**181**
Hexan-2-ol	–	–	–	–	–	–	6.37[b]	–
Heptan-2-one	–	15.4	35.5[c]	27.0	20.1[b]	66.6	119	**679**
Heptan-2-ol	–	5.26[b]	–	–	–	8.27[b]	**27.2**[c]	10.6[c]
Octan-2-one	–	33.8	81.3	–	–	38.5	207	**808**
Octan-2-ol	–	13.5[b]	9.47[c]	–	–	21.5[b]	32.6[c]	**41.0**[c]
Acetophenone	4.67[c]	20.5	6.43[c]	49.9	45.3	**1620**	106	149
4-Methylphenol	3.34[c]	243	2290	763	1910	3640[c]	3500	**11,300**
Nonan-2-one	2.50[b]	25.6	54.3	52.0	15.2[b]	1260	4970	**12,300**
Nonan-2-ol	–	–	–	113	60.8	727[c]	**1600**[c]	194
Decan-2-one	–	–	9.57[b]	1.74[b]	–	104[c]	232	**1840**
Decan-2-ol	–	–	–	3.67[b]	–	90.5[b]	**648**[c]	3.63[b]
4-Ethylphenol	–	28.8[c]	168[c]	127	165	**1110**[c]	483	707
Undecan-2-one	–	4.73[b]	22.0[c]	–	–	352	2050	**4440**
Undecan-2-ol	–	–	–	14.5[b]	–	362[b]	**1300**[c]	28.6[b]

The maximum relative value for each compound is indicated in bold. All data were acquired 48 h after thawing. Unless otherwise noted, means are based on data from all three elephants. [CF = centrifuged and filtered; CFNMP = non-musth centrifugation pellet introduced; CFMP = musth centrifugation pellet introduced; CFA = autoclaved centrifugation pellet introduced; CFF = feces introduced]
[a]A dash indicates non-detectable or inadequate amount for accurate integration, [b]$N = 1$,[c]$N = 2$

urine with the following additions made before aging: autoclaved musth urine centrifugation pellet (CFA) or fresh African elephant fecal bacteria (CFF).

The results in Table 6.1 show the following:

- The compounds of maximum abundance in general were nonan-2-one and 4-methylphenol
- No compound exhibited its maximum abundance in either non-musth urine, the centrifuged and filtered musth urine, or the centrifuged and filtered musth urine to which the autoclaved centrifugation pellet was added (indeed, the largest number of undetected compounds was clearly for the CF non-musth samples)
- With the exception of pentan-2-one, the maximum abundance for the alkan-2-ones was evident in the CFMP samples
- The only compounds exhibiting maximum abundance in the CFNMP were four alkan-2-ols
- In all cases, the combined abundance of an alkan-2-one and the corresponding alkan-2-ol was highest in the CMFP samples
- For all musth compounds, the abundance was higher in the CFF samples than in the CF or the CFA samples

Table 6.2 Comparison of selected compounds in musth and non-musth urine of a male Asian elephant under various conditions. The data are derived from peak areas from GC-MS total ion chromatograms normalized to the value for non-musth CFNMP octan-2-one, the compound of lowest abundance[a]

	Non-musth			Musth				
Compound	CF	CFNMP	CFMP	CF	CFA	CFF	CFNMP	CFMP
Heptan-2-one	4.03	3.39	15.7	13.8	13.0	**37.9**	18.6	13.1
Heptan-2-ol	–	–	–	–	–	**3.93**	–	2.15
Octan-2-one	4.84	1.00	**46.2**	–	–	15.2	3.40	7.16
Octan-2-ol	–	–	16.9	–	–	–	–	–
Acetophenone	16.4	20.2	12.0	58.6	68.7	**264**	73.8	52.6
4-Methylphenol	–	–	**2890**	207	419	269	1050	1210
Nonan-2-one	–	–	53.7	3.23	2.43	**253**	140	241
Nonan-2-ol	–	–	11.3	2.44	5.67	2.43	43.2	**53.4**
1-Phenylpropan-2-one	–	–	–	123	0.81	**139**	22.1	71.5
Decan-2-one	–	–	**54.3**	–	–	–	4.40	–
Decan-2-ol	–	–	–	–	–	–	–	–
4-Ethylphenol	–	–	–	–	5.31	**19.9**	–	1.81
Undecan-2-one	–	–	–	–	-	15.8	**40.7**	24.5
Undecan-2-ol	–	–	–	–	–	–	3.72	**7.08**

[a]A dash indicates non-detectable or inadequate amount for accurate integration

The maximum relative value for each compound is indicated in bold. All data were acquired 48 h after thawing. [CF = centrifuged and filtered; CFNMP = non-musth centrifugation pellet introduced; CFMP = musth centrifugation pellet introduced; CFA = autoclaved centrifugation pellet introduced; CFF = feces introduced]

The results in Table 6.2 show the following:

- The compounds of maximum abundance were nonan-2-one and 4-methylphenol.
- Most compounds exhibited their maximum abundance in musth urine samples; however three compounds exhibited maximum abundance in the non-musth CFMP samples.
- The largest number of maximum compound abundances were in the musth CFF samples.

6.3.3 Relative Abundance of Medium- and Long-Chain Fatty Acids in Aged Elephant Urine

As will be detailed in the Discussion, we believe that the exogenously produced urinary alkan-2-ones are the result of bacterial metabolism of fatty acids, and that the alkan-2-ols are derived by reduction of the alkan-2-ones. The urine samples were analyzed on the day of thawing from −70 °C and were kept at the ambient temperature until the sixth day after thawing and analyzed once more. As shown in Table 6.3, 10 fatty acids were identified. The highest abundance for each acid is bolded.

6.3.4 African Elephant Urinary Proteins

Alignment of the*L. africana* (G3SMX8) and *E. maximus* (Q6B3Z0; Lazar et al. 2004) albumin sequences from the UniProt database showed sequence homology of over 99 % between the two species. A glycoprotein (a uromodulin; Devuyst et al. 2005) from African elephant urine was also sequenced (accession number G3SN28).

6.4 Discussion

6.4.1 Introduction

As previously stated, Rasmussen and Wittemyer (2002) found that a series of alkan-2-ones were present in higher concentration in the urine of wild African elephants in musth when compared to non-musth urine. Rasmussen and co-workers also found volatile ketones emanating from the temporal gland orifices (prior to drainage), temporal gland secretions (TGS), breath, and urine of captive and wild musth Asian elephants (Rasmussen et al. 1990; Perrin et al. 1996; Rasmussen and Perrin 1999). Similar sets of alkan-2-ones and the corresponding alcohols were present in higher abundances in musth urine of captive African elephants, and the

Table 6.3 Relative fatty acid abundances (mmol/mol creatinine as determined by GC-MS using an internal standard) from musth and non-musth urine of 3 African and 1 Asian elephants (#4)

Elephant	Hexanoic (D1) C-6	Hexanoic (D6)	Octanoic (D1) C-8	Octanoic (D6)	Capric (D1) C-10	Capric (D6)	Lauric (D1) C-12	Lauric (D6)	Myristic (D1) C-14	Myristic (D6)
#1 M	11.30	19.30	17.30	**25.70**	1.20	1.00	13.80	**52.50**	1.00	–
#1 NM	4.70	3.10	24.70	20.70	1.70	2.30	20.80	31.30	–	–
#2 M	7.00	11.40	11.30	5.80	2.00	2.50	29.70	25.10	10.10	–
#2 NM	5.80	7.90	12.60	13.40	1.60	1.90	25.60	28.50	3.80	–
#3 M	10.20	12.20	14.30	12.20	2.30	3.20	14.70	18.30	**19.30**	1.00
#3 NM	8.40	9.90	14.50	15.70	2.80	4.80	26.20	21.60	5.50	1.90
#4 M	5.00	4.90	3.80	4.60	1.70	1.60	34.10	43.60	7.40	–
#4 NM	28.00	**28.90**	16.00	23.00	**5.70**	4.70	18.60	11.40	9.10	5.40
Elephant	Palmitic (D1) C-16	Palmitic (D6)	Oleic (D1) *18:1 *cis*-9	Oleic (D6)	Linolenic (D1) *18:3 *cis*,*cis*,*cis*-6,9,12	Linolenic (D6)	Linoleic (D1) *18:2 *cis*,*cis*-9,12	Linoleic (D6)	Stearic (D1) C-18	Stearic (D6)
#1 M	6.80	7.70	11.10	24.60	–	–	7.90	4.80	10.80	11.10
#1 NM	8.30	7.30	12.80	22.60	–	–	9.30	5.40	15.20	11.70
#2 M	14.50	13.90	35.00	44.90	–	29.30	23.40	11.40	24.90	21.80
#2 NM	14.00	12.40	21.00	40.20	–	–	18.80	8.40	26.80	19.00
#3 M	16.10	**89.00**	21.40	**128.10**	**46.30**	40.50	22.60	**181.90**	22.60	**190.70**
#3 NM	36.70	46.60	40.50	117.90	–	–	92.60	72.50	77.10	101.40
#4 M	24.80	19.90	27.90	41.90	–	–	58.80	25.80	53.30	37.40
#4 NM	60.60	38.70	73.70	107.70	–	–	121.70	42.20	131.80	73.30

The data are normalized to the smallest number. (D1 = day of thawing; D6 = 6th day after thawing; C-X = number of carbon atoms; lipid numbers* are given for the unsaturated fatty acids: oleic, linolenic, and linoleic). The highest number for each acid is bolded

abundances increased exogenously when the urine was aged at the ambient temperature (Goodwin et al. 2012).

Musth Asian males were also found to exhibit a large elevation of serum triglycerides and the lipase that catalyzes their hydrolysis to fatty acids and glycerol, leading to speculation that the alkan-2-ones are derived from fatty acids (Rasmussen 1999; Rasmussen and Perrin 1999). If this hypothesis is supported, then two questions naturally arise: (1) By what biochemical pathway are the alkan-2-ones synthesized from fatty acids? (2) Why are serum levels of fatty acids and urine levels of alkan-2-ones particularly elevated during musth? Both questions are addressed below, as is the role of bacteria in the continued urinary production of alkan-2-ones and the corresponding alkan-2-ols exogenously as the urine ages.

6.4.2 Biosynthesis of Alkan-2-Ones from Fatty Acids During Musth

Adrenalin (epinephrine) induces increased mobilization and lipolysis (hydrolysis) of adipose tissue triglycerides during exercise (Okuda 1975; Horowitz 2003). Analogously, malnutrition and untreated diabetes generate a need for energy derived from metabolism of stored triglycerides to replace energy normally supplied by carbohydrate metabolism (Miaskiewicz et al. 1989; Stryer 1995, p 612; Arner 1995). At such times some acetyl CoA, the usual end product of fatty acid metabolism, is diverted from the citric acid cycle to be used in biosynthesis ("ketogenesis") of the so-called ketone bodies (acetoacetate, acetone (propan-2-one), and β-hydroxybutyrate), which appear in the urine. Ketogenesis begins via condensation of two acetyl CoA subunits to produce acetoacetyl CoA, which is subsequently transformed into acetoacetate. β-Hydroxybutyrate and acetone are produced from acetoacetate by carbonyl reduction and decarboxylation, respectively (McClatchey 2002, p 526–527; Nelson and Cox 2008, p 666–667). Thus, metabolism by this secondary pathway can explain the increased production of propan-2-one by musth elephants.

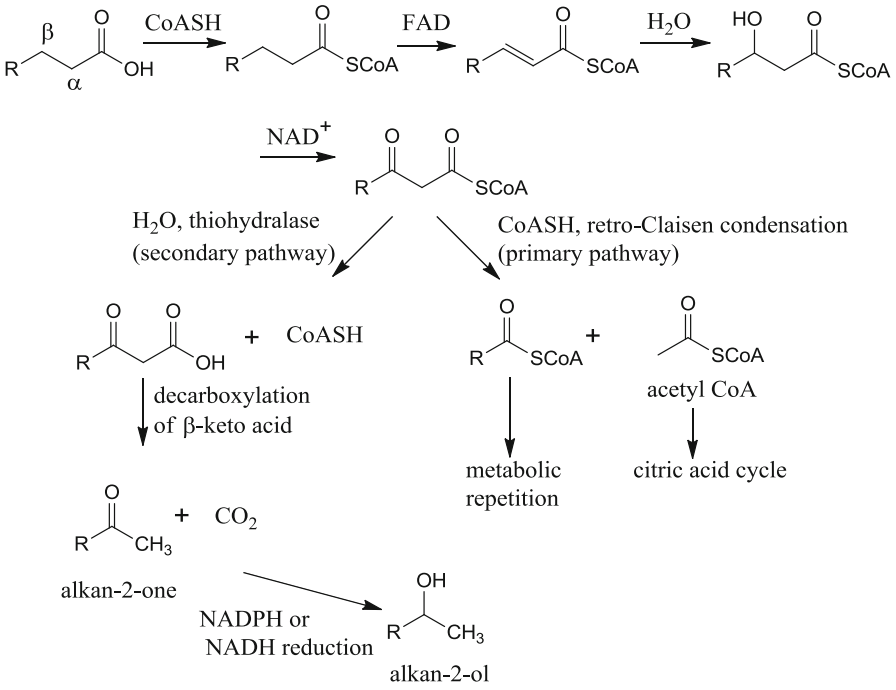
Butan-2-one synthesis can occur in an analogous manner via condensation of acetyl CoA with propionyl CoA, a residue of catabolism of fatty acids with an odd number of carbon atoms (Stryer 1995, p 612); yet this type of process may not fully account for the biosynthesis of higher ketone homologs. The production of higher homologs of propan-2-one in plants via decarboxylation of β-keto acids was proposed long before the chemistry of "ketone body" formation was well understood (Dakin 1908). Much later, Rhodes et al. (1982, p 39) detected alkan-2-ones in the urine of diabetic rats and proposed their genesis from "decarboxylation of keto acids, in a manner similar to the well-known formation of acetone" (from acetoacetate). This was also demonstrated to be the likely pathway by which alkan-2-ones are generated by microorganisms from milk fats in cheese (McSweeney and Sousa 2000). Interestingly, some of propan-2-one's higher

homologs (pentan-2-one, heptan-2-one, nonan-2-one, and several branched-chain, low-molecular-weight alkan-2-ones) have been detected as volatile metabolites in normal and diabetic human urine, possibly arising by the β-keto acid decarboxylation pathway (Zlatkis et al. 1973; Mills and Walker 2001).

Rasmussen and Perrin (1999) were the first to suggest that high levels of propan-2-one and a few other ketones in the breath of Asian elephants in musth were indicative of lipid catabolism. Rasmussen also proposed that decanoic acid might be converted into nonan-2-one, a compound identified in the temporal gland secretion of an Asian elephant in musth (Rasmussen et al. 1990; Rasmussen and Krishnamurthy 2000). In this and similar studies, nonan-2-one was invariably the third most abundant urinary alkan-2-one, with only propan-2-one and butan-2-one being present in higher concentrations (Rasmussen and Wittemyer 2002). Analogously, Novotny and co-workers (2007) found heptan-2-one to be a urinary chemical signal in female mice (*M. musculus domesticus*), and attributed its metabolic origin and that of similar compounds to fatty acid oxidation (see also Zhang et al. 2008).

Fatty acid metabolism ("the β-oxidation pathway") usually proceeds via a β-keto thioester which reacts with CoASH in a retro-Claisen condensation to provide one equivalent of acetyl CoA and a thioester which has been shortened by two carbons, and this process is repetitive (Scheme 6.1, primary pathway; Stryer 1995, p 605–611; Kunau et al. 1995; Laposata 1995; Bhaumik et al. 2005). However, when sufficient energy cannot be produced from carbohydrate metabolism (as with diabetes, malnutrition, or excessive exercise), unusually large quantities of stored triglycerides are hydrolyzed and released from adipocytes into the bloodstream as fatty acids. Elevated adrenaline and androgen levels also initiate mobilization of stored fatty acids into the bloodstream. When fatty acid concentrations overwhelm the ability of the normal catabolic production of acetyl CoA to keep up, a secondary pathway is activated. This process involves hydrolysis of the intermediary β-keto thioester to a β-keto acid which decarboxylates to produce an alkan-2-one with one less carbon (Scheme 6.1, secondary pathway). Because successive iterations of the primary pathway can produce a series of β-keto acids each two carbons shorter than its predecessor, a series of alkan-2-ones can result by the secondary pathway from metabolism of long-chain fatty acid precursors. The fatty acids or triglycerides can be of dietary origin, or may be synthesized in vivo from acetyl CoA (Stryer 1995, p 614–618; Laposata 1995).

There is good experimental evidence for the operation of this secondary pathway (Scheme 6.1) to produce alkan-2-ones (and thence reduction to the corresponding alkan-2-ols; Scheline 1973; Schulz and Dickschat 2007), notably in the catabolism of milk fatty acids by microorganisms to give a characteristic flavor and odor to certain cheeses (Lawrence and Hawke 1966; Kinsella and Hwang 1976a, b; McSweeney and Sousa 2000; Marilley and Casey 2004). Additionally, this pathway for biosynthesis of alkan-2-ones from fatty acids has been demonstrated to be operative in a wild tomato species (Fridman et al. 2005) and marine arctic bacteria (Dickschat et al. 2005).



Scheme 6.1 Two pathways for fatty acid metabolism via β-oxidation

We suggest that the secondary biosynthetic pathway (Scheme 6.1) is followed for the production of alkan-2-ones and alkan-2-ols endogenously in elephants, and is followed exogenously as well in the urine via bacterial fermentation of fatty acids. It is likely followed in other mammals as well. For example, in the breeding season the urine of the male brown antechinus (*Antechinus stuartii*), a small Australian marsupial, contains a series of alkan-2-ones (hexan-2-one, octan-2-one, nonan-2-one, decan-2-one, and undecan-2-one) that are not present in the urine of females or castrated males (Toftegaard et al. 1999). The brown antechinus is one of only a few mammals (all in the Dasyuridae and Didelphidae families) in which the males die after one breeding season (i.e., they are semelparous). The cause of death is the deleterious effect of unabated high levels of cortisol and testosterone (Naylor et al. 2008).

6.4.3 Proximate Causes of Elevated Fatty Acid and Alkan-2-one Levels During Musth

A study on male moose (*Alces alces*) in rut provides experimental findings that are relevant to understanding certain aspects of elephant musth (Miquelle 1990). Mature male moose cease feeding completely (hypophagia) for about 2 weeks during their rutting period, and a similar appetite suppression is common for the rut in males of

other ungulate species (Coblentz 1976). During the rut, male moose use urine marks to attract female conspecifics, and possibly to induce ovulation (Miquelle 1991). The peak timing of hypophagia coincides with maximum urine marking. A common proposal for elephants during musth is that decreased feeding and the resultant weight loss result from a high expenditure of energy as males search for and breed females, therefore having little time for foraging (Barnes 1982; Poole 1989). Miquelle (1990) found that during the rut, male moose spend approximately 45 % of their time standing inactive, and therefore have abundant free time to eat should they so desire. He proposed the following (p 150): "… hypophagia is a byproduct of a physiological process associated with scent-urination." There are good reasons to suggest that this physiological process is the increase in serum androgen concentrations and subsequent metabolism of an abundance of lipids as discussed below.

The androgen dependence of the chemical composition of male rodent urine during scent marking has been conclusively demonstrated (Roberts 2001). The increased mobilization of fatty acids into the bloodstream during moose rut and elephant musth is likely stimulated by the elevated level of androgens as well. This phenomenon has been proposed for elephants (Poole 1989; Rasmussen and Perrin 1999) and is well documented in humans (Kartin et al. 1944; Chioléro et al. 1997). Elevated androgen levels can lead to increased serum fatty acid concentrations through de novo lipogenesis from acetyl CoA, as well as from mobilization and lipolysis of stored triglycerides from adipose tissue (Swinnen and Verhoeven 1998; Saleh et al. 1999). Therefore it is reasonable to propose that male elephants decrease foraging and lose weight during musth not merely because they are busy pursuing females, but also due to elevated androgens and subsequent catabolism of triglycerides. In fact, Poole (1989, p 147) notes that captive Asian elephants have been reported to "lose weight during musth even when they are chained and given normal rations of food" (Deraniyagala 1955).

6.4.4 The Role of Bacteria in Chemical Signals of Elephant Musth

What we know:

- The ability of bacteria to produce alkan-2-ones and alkan-2-ols has been demonstrated previously (Dickschat et al. 2005; Schulz and Dickschat 2007), as well as in our preliminary studies when we inoculated urine with pure strains of bacteria (Goodwin, unpublished).
- There are bacteria in elephant urine (Sects. 6.2.4 and 6.2.5; Fig. 6.1).
- Musth male elephant urine has elevated levels of alkan-2-ones and alkan-2-ols compared to non-musth urine (Rasmussen and Wittemyer 2002; Goodwin et al. 2012; Tables 6.1 and 6.2).
- The abundance of these urinary ketones increases exogenously as the urine ages (Goodwin et al. 2012).

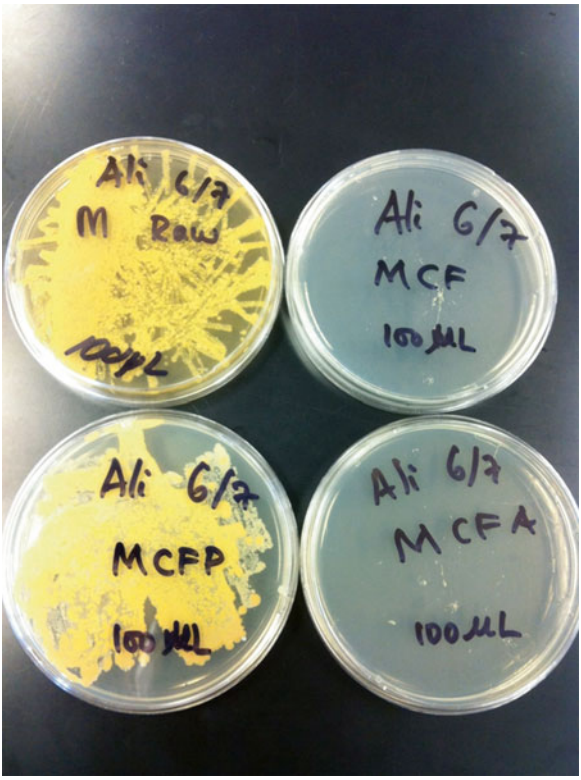


Fig. 6.1 Cultures of bacteria from musth urine. *Upper left*: Untreated musth urine; *lower left*: centrifuged and filtered, musth pellet reintroduced; *upper right*: centrifuged and filtered; *lower right*: centrifuged and filtered, autoclaved pellet reintroduced

- The exogenous production these compounds can be stopped by centrifuging (10–15,000 × *g* for 2 min) and filtering (0.22 μm sterilizing filter to remove insolubles, including microbes) (Tables 6.1 and 6.2; Fig. 6.1).
- We can restore the exogenous production of these compounds by reintroducing the centrifugation pellet (unless the pellet is autoclaved first) and by inoculation with feces.
- The ability of microbes to convert fatty acids to alkan-2-ones and alkan-2-ols has been demonstrated (Lawrence and Hawke 1966; Kinsella and Hwang 1976a).
- Elephant urine contains fatty acids (Table 6.3).
- Elephant urine contains albumin (Sects. 6.2.7 and 6.3.4).

6.5 Conclusion

In Sects. 6.4.2 and 6.4.3 we discussed proximate causes for higher levels of serum fatty acids during elephant musth, and how this could lead to the higher levels of alkan-2-ones and alkan-2-ols in musth urine. We also proposed that as the expelled

urine aged, bacterial metabolism of urinary fatty acids leads to the observed increase in abundance of these ketones and alcohols. An obvious explanation for higher levels of alkan-2-ones and alkan-2-ols in musth urine versus non-musth urine would be that the concentrations of their fatty acid precursors are higher in musth urine, but that is not evident from the data in Table 6.3 (although a larger data set might be more revealing). There is yet another compelling explanation, as discussed below.

We propose that the fermentation hypothesis (Albone 1984) is operative in chemical signaling of elephant musth. Of particular interest for the present study is Albone's prescient statement that one function of microbial systems in mammalian scent production requires time modulation of fermentation product composition: "… as in signaling the occurrence of a particular physiological state in a mammal. The mammal is able to control the fermenting system by varying substrate composition and availability, and providing that microorganisms are present which have adapted to utilize the new substrate, the response in terms of signal production will be rapid" (Albone et al. 1977, p 38). Theis et al. (2013, p 19835) suggest a general "symbiotic hypothesis," the explanatory potential of which is "limited only by the capacity of hosts' social and physiological circumstances to alter the structure of their symbiotic microbial communities in ways that consequently affect hosts' odor profiles in signaling-relevant ways."

Thus elephants in musth may have a urinary microbial community that is somehow different from that when not in musth. This could explain why adding a musth urine centrifugation pellet (presumably containing bacteria) to centrifuged and filtered musth and non-musth urine generally results in higher abundances of alkan-2-ones and alkan-2-ols than does addition of a non-musth centrifugation pellet (Tables 6.1 and 6.2). It is also reasonable to propose that bacterial communities in elephant urine derive, at least in part, from the distal urethra, periurethral area, and/or the penile sheath (Spainea et al. 2000). Of interest in this regard is that of the two bacteria mentioned in Sect. 6.2.5, one matched most closely in the BLAST search to *Micrococcus xinjiangensis*, and the other to *Staphylococcus sp*. These are common types of skin bacteria (James et al. 2013).

So what directions might this field of research go in the future? Consider a recent review on microbially produced chemical signals in animals, in which Ezenwa and Williams (2014, p 3) stated the following": Mammals are the most-studied taxon in terms of microbial-based olfactory signaling, yet evidence from this group is still rather weak in two respects. First, only in a few cases have cause and effect relationships been established. Second, the specific microbes involved are typically unknown." These authors state, in specific reference to our elephant research (Goodwin et al. 2012), that "… captive individuals could be used to test for effects of microbial addition on receiver behavior." Thus behavioral bioassays could be devised in which responses to centrifuged and filtered elephant urine are compared to responses to untreated urine, and also to study whether responses change as the urine samples age. Additionally, as all of the selected ketones and alcohols are commercially available, mixtures of these could be bioassayed. In addition, the bacterial communities in elephant urine can be thoroughly surveyed as recently demonstrated with hyena secretions (Theis et al. 2013). In preliminary studies we

have observed the ability of pure bacterial strains isolated from elephant urine to produce alkan-2-ones and alkan-2-ols when introduced to centrifuged and filtered elephant urine (Goodwin, unpublished), thus this avenue of research also deserves further attention.

The field of microbial chemical ecology is burgeoning as evidenced by a special issue of the *Journal of Chemical Ecology* (2013, 39:807–1054), yet none of the excellent articles contained therein focused on the role of microbes in mammalian chemical signaling. Many years ago Albone et al. (1977, p 35) wrote the following: "Although it has long been known that microorganisms are, at least partially, responsible for odor, such as axillary odor and halitosis, or breath odor, in man, only recently has it been suggested that microbially-derived odors might assume a chemical significance in mammals." Much has changed in the ensuing years as procedures, techniques, and instrumentation have improved dramatically, and as microbiologists, behavioral ecologists, chemists, and others have contributed new knowledge regarding the role of microbes in mammalian semiochemistry. We are glad that the majestic elephants and their tiny symbionts have a role to play in this fast-moving and fascinating field of research.

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