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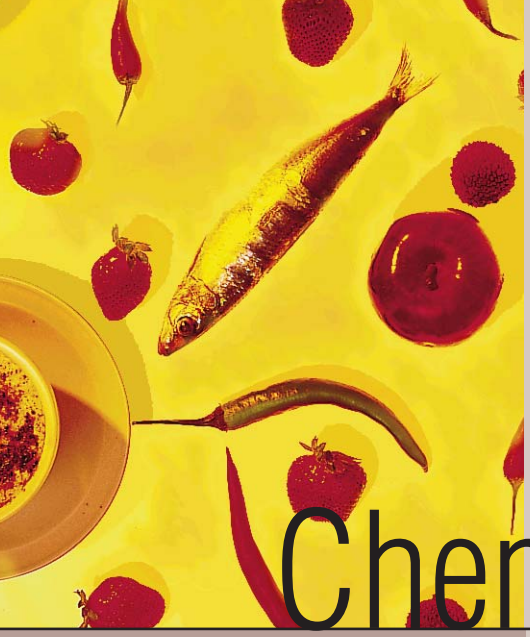

ChemoSense

Editorial

By Graham Bell
g.bell@atp.com.au

The Bets Rasmussen Issue

Although it is over two years since the flame of life was extinguished in Bets Rasmussen, her legacy as scientist blazes on. This issue is dedicated to Bets and to her achievements. It carries an interesting dedication about her as a person, and two significant contributions from her colleagues, in which the elephant, Bets's animal of choice, again comes under scientific scrutiny and we learn more about the workings of the mammalian olfactory system.

Wendy Parr takes us to New Zealand, and her home region of Marlborough, where wine, however it is or will be labelled (Wendy explains all this), continues to wow the wine world.

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The Elephant as an ideal olfactory model mammal

David R. Greenwood[1,2] and L.E.L. "Bets" Rasmussen[3,4]

1. New Zealand Institute for Plant and Food Research Limited (Plant & Food Research), Mt Albert Research Centre, Private Bag 92-169, Auckland 1142, New Zealand
2. Centre for Genomics and Proteomics, School of Biological Sciences, University of Auckland, Private Bag 92-019, Auckland 1142, New Zealand
3. Department of Environmental and Biomolecular Systems, OGI School of Science and Engineering, OHSU, Beaverton OR, USA
4. Deceased, September 2006

Background:

The last 25 years has been a productive time for studies on olfaction and chemosensory science, helped in no small way by technological developments in the analysis of volatile compounds by solid-phase microextraction (SPME) and capillary gas chromatography mass spectrometry (GCMS). While the next decade promises to be equally intense due to the explosion of sequenced and annotated genomes as researchers delve into the intricacies of olfactory receptor structure and signalling systems, there remains a chasm that few groups have tried successfully to bridge – that of defining the actual role of individual compounds in semiochemical-mediated

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INSIDE:

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EDITORIAL CONTINUED

Editorial continued

The Australasian Association for ChemoSensory Science (AACSS) invites you to its next scientific meeting on the Australian Great Barrier Reef, from 2-6 December 2009. ■

The Elephant as an ideal olfactory model mammal continued

behaviour in higher animals, especially in mammals. Sure, there have been many successes from delving into the world of invertebrate pheromones and olfaction, but surprisingly few cases have amassed from higher organisms for reasons that will become apparent below.

The role of behaviour as a tool for guiding bioassay-directed fractionation of semiochemical-containing extracts is now a well-defined and particularly successful approach for identifying the individual components and active blends involved in chemical signalling in animals, including mammals. However, the underlying complexity and subtlety of any elicited behavioural responses can blur and effectively complicate interpretation. This is manifested by the surprisingly few cases where individual compounds and/or blends have been defined as eliciting key response traits in receiving higher organisms, especially mammals. Our contention is that there are distinct reasons why this is the case and we offer a solution in part to help address the problem.

Professor Rasmussen and her extensive network of collaborators over the last 20+ years have defined the chemical composition of secretions eliciting several key behavioural traits particularly of the Asian elephant *(Elephas maximus)*. We attribute much of this progress to the Asian elephant itself and a specific set of factors, several unique to this species. While the African elephant *(Loxodonta africana)* is similar in many respects, the semiochemistry of the Asian counterpart has so far proven the easier of the two to decipher. We suggest therefore that the Asian elephant is an ideal olfactory model for mammalian olfaction for the following compelling reasons:

Scenario:

1. **Two discreet pheromones have been identified:**

Two sexually dimorphic pheromones — a preovulatory urinary pheromone, (Z)-7-dodecen-1-yl acetate (Z7-12:Ac) (Rasmussen, 2001; Rasmussen et al., 1996; 1997; 2005), and a musth temporal gland secretion pheromone, frontalin (Greenwood et al., 2005a; Rasmussen & Greenwood, 2003), which on their own and without additional components **(Fig.1)**, elicit key behaviours that are likely to be central to reproductive success of this species. These compounds meet the definition of a pheromone (Beauchamp et al., 1976; Johnston, 2000; Karlson & Luscher, 1959).

The titre of Z7-12:Ac increases steadily in the blood of oestrous females to a peak immediately prior to ovulation. This blood titre is mirrored by excretion of Z7-12:Ac in the urine up to millimolar concentrations, the pheromone being almost entirely bound to the protein albumin, the secretion of which through the kidney is interesting in its own right. A female at ovulation can conceive over only a narrow 24 hr window. When one considers the fact that the sexes live apart in same sex groups for most of their lives, coming together only for reproduction, demonstrates the importance of chemical cues in the reproductive cycle.

Males on the other hand secrete the bicyclic monoterpene acetal pheromone frontalin, at the time of musth through the temporal gland (unique to elephants on the side of the head) as well as in the urine. The titre of frontalin correlates with the period of musth, a cyclical condition of raging testosterone levels and heightened sexual interest. It tracks the increasing level of maturity of the male through successive musth bouts. Frontalin exists as two chiral forms and for maximal effect, the ratio of the (+) and (–) enantiomers must be in approximately a 1:1 ratio (Greenwood et al, 2005a). This racemic mix is found consistently in mature males, the ratio of the two forms in

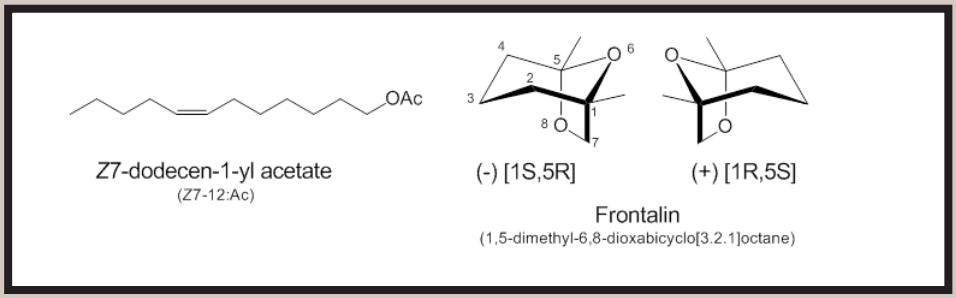

Figure 1. Asian elephant pheromones: (Z)-7-dodecenyl acetate (Z7-12:Ac), the female-to-male sex pheromone, and frontalin in its enantiomeric forms, the male dominance pheromone.

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Air quality and human health

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Preliminary program

8.00 Light breakfast for delegates and exhibitors

8.30 **EcoForum keynote address** Why environmental behaviour changes away from home Prof. Joe Arvai *Michigan State University USA* e9167

Odour analysis

9.30 **Stream keynote address** Human olfaction Prof. EP Köster *Utrecht University* The Netherlands e9144

10.00 Can statistics help environmental analysis? Brynn Hibbert *University of NSW* e9142

10.30 – 11.15 Morning refreshments

Human health and vapour intrusion

11.15 Health screening levels for petroleum hydrocarbons being developed by CRC CARE—project update Eric Friebel *GHD* e9028

11.25 Risk management at residential and commercial sites impacted by methane and other landfill gases Chris Jewell *C M Jewell & Associates* e9054

11.35 Assessment of human health risk from vapour intrusion into residential dwellings Adrian Heggie *Parsons Brinckerhoff* e9036

11.45 Modelling and managing vapour intrusion—balancing risk and commercial considerations Chris Jewell *C M Jewell & Associates* e9056

11.55 Review of data for assessing vapour intrusion on 47 petroleum sites in Australia Christina Robinson *URS Australia* e9097

12.05 **EcoForum discussion** e9184

12.45 – 2.15 Lunch

Odour measurement

2.15 Selection of effective sampling methods to refine health risk assessments Judith Barnes *URS Australia* e9083

2.25 Biofilter design and operation in 2008—a technology review Terry Schulz *The Odour Unit* e9131

2.35 Improving odour management using olfactory GC-MS Richard Stuetz *University of NSW* e9133

2.45 Development of e-noses for real-time recognition of complex odours regarded as nuisances by local communities Graham Bell *E-Nose* e9118

2.55 Measurement of chemical emissions from building products Robert Schiller & Subbalakshmi Yerramilli *Cetec* e9062

3.05 BIOVAPOR—a spreadsheet model for evaluation of vapour intrusion with oxygen-limited biodegradation Curtis Stanley *Shell Global Solutions* e9119

3.15 **EcoForum discussion** e9183

3.45 – 4.30 Afternoon refreshments

Air quality—can we do better?

4.30 Indoor air quality, odours and ventilation energy use Paul Spry *Spry Consulting* e9143

4.45 **EcoForum discussion** Reviewing the data discussed today Graham Bell *E-Nose* e9143

5.30 Happy hour

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The Elephant as an ideal olfactory model mammal

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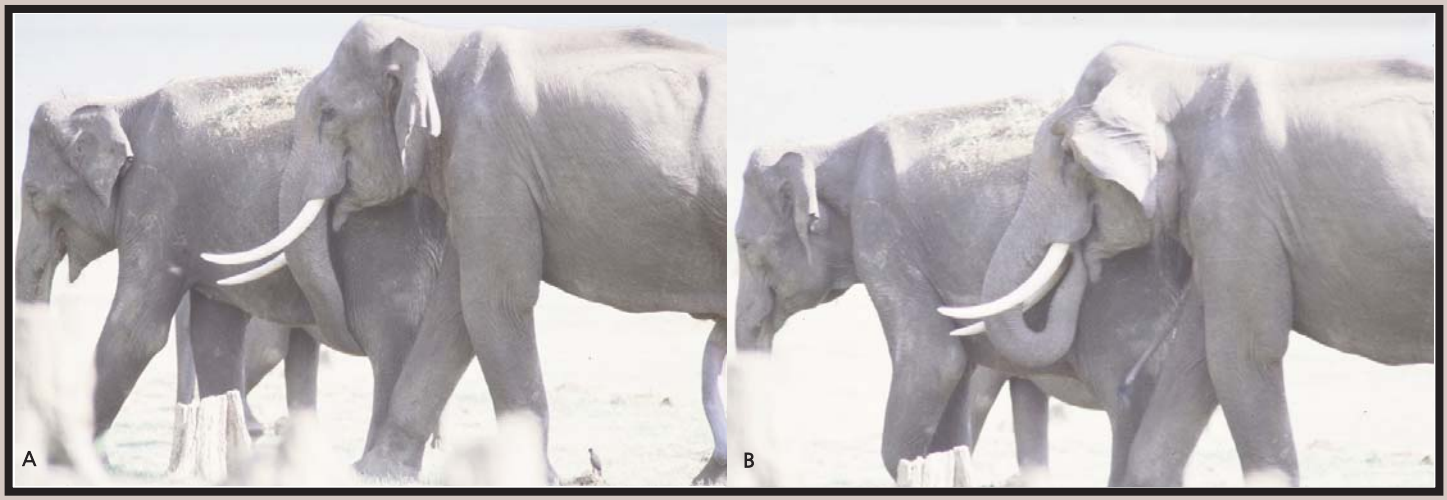

Figure 2. (A) A wild male Asian elephant samples the urogenital region of a female with his trunk tip, and (B) performs a flehmen to pass the chemical signal to his vomeronasal organ to assess her readiness to mate.

younger males fluctuating wildly (L.E.L. Rasmussen & D.R. Greenwood, unpublished). These differences are detected by all conspecifics, such that ovulating females are attracted to mature males in musth whereas other elephants are generally repelled or are indifferent. Hence racemic frontalin serves a spatial segregating role, essentially as a dominance pheromone, helping minimise competition for females while concomitantly assisting in self preservation, since aggressive encounters are avoided or at least minimised by secreting this assertive chemical signal. In this way frontalin in its two forms differentially modulates behavioural activities depending on gender and life stage (Rasmussen & Greenwood, 2003).

2. Behaviours correlate with chemosensory responses to pheromones:

Both pheromones, Z7-12:Ac and frontalin, elicit chemosensory responses that are clearly observed and enumerated with behavioural consequences that are relevant to elephant life scenarios (Rasmussen & Greenwood, 2003; Rasmussen & Krishnamurthy, 2000; Rasmussen et al., 1997; 2002; 2005). The underlying key to the success of this work has been the separation of volatile olfactory and pheromonal olfactory modes by virtue of the elephant's unique anatomy and large size that effectively separates spatially and temporally the various chemosensory responses for the observer to interpret. Quantification of these responses is straight forward with much of the action being concentrated around the readily decipherable flehmen **(Fig.2b)**, the act of transferring samples to the vomeronasal organ (VNO). This involves the trunk tip being daubed into the sample, be it urine spot, female urogenital region **(Fig.2a)**, or male facial temporal gland. The sample is mixed with copious trunk mucus and the wet tip curled upwards and deposited on the entrance to the VNO ducts: paired portals on the roof of the mouth **(Fig.3)**. There is then a time lag of several seconds as the sample travels the length of the ciliated VNO duct, posteriorally into the VNO itself where specialist olfactory receptors reside to detect any pheromone signal. We believe an elephants quantitatively assesses the amount of signal present as they will often resample at this point after which the animal normally makes a clear behavioural decision based on this sampling regime. Data from many hours of watching, videotaping and replaying to enumerate the various categorised responses of wild and captive animals result in robust conclusions as to the relative activity of crude extracts and pure and admixture compounds.

Figure 3. The open mouth of a female Asian elephant showing the position of the paired VNO duct portals.

3. Partial deciphering of the perireceptive system:

Over the last ten years considerable progress has been made in determining the respective flow paths of both pheromones from 'source-to-sink', i.e. from the sending individual to the recipient. For the two pheromones binding to proteins notably serum albumin has been demonstrated at source in elephant blood and in the various secretions and excretions following exit from the body (Lazar et al., 2004; D.R Greenwood and L.E.L. Rasmussen, unpublished results). This presumably serves to reduce loss of pheromone

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The Elephant as an ideal olfactory model mammal

continued

by volatility from these materials, which may remain undetected for hours if not days. On sampling by passing elephants, the transfer phase occurs which involves mixing the voided materials with acidic trunk mucus, where the compounds are released in a pH-mediated transfer from albumin to then be scavenged by lipocalins, 18 kDa ligand binding proteins which abound in the trunk mucus (Lazar et al., 2002; D.R. Greenwood & L.E.L. Rasmussen, unpublished results). These latter pheromone binding proteins bind both compounds. A certain amount of this released pheromone will remain free (unbound) and is probably detected by the normal olfactory system via the trunk but more importantly also the VNO.

Initial kinetic studies performed with Z7-12:Ac have challenged previous dogma that pheromone binding proteins are not in fact acting as transporters, but rather are sequesters of pheromone, mopping up excess as a result of tight binding (extended off rates, $T_{1/2}$ = 30 min) and the high pheromone titre observed (Lazar et al., 2002). The pH-mediated changes in protein binding affinities provide an ideal explanation for the flux of the respective pheromones during the transfer process. We are now poised to study what happens within the VNO duct as the pheromone molecules travel the length of the duct to the receptor proteins in the VNO itself for detection. We have evidence for another pheromone binding protein present in the residing ductal mucus (Greenwood & Rasmussen, 2003; D.R. Greenwood & L.E.L. Rasmussen, unpublished results).

4. Innovative visualization tools:

Synthetic forms of both Asian elephant pheromones are available (as insect sex attractants from chemical companies specialising in synthesising non-toxic insect control agents) and analogues including radioactive and photoactivatable forms have been developed for competitive studies that retain biological activity (Prestwich, 1991; Greenwood et al., 2005b; 2005c). Moreover the ability to synthesise specific enantiomers of frontalin in high enantiomeric enrichment (Mori, 1975; Lee, 1983) means that we can potentially examine the interplay between receptors to examine how the two forms of frontalin are detected and to rationalise the coordinated response to racemic frontalin.

5. Mammalian model with a highly developed society, yet maintaining pheromone utilisation:

To date, no other mammal with such a highly developed society, organized for complex life situations and displaying such high-level cognitive abilities (i.e. long-term memory and associative skills) has had its primary pheromonal systems identified. Moreover that these are accompanied by discrete responses and behaviours which are robustly quantifiable is an obvious boon for those experimenters considering extending to more subtle chemosensory examples.

6. Decipherable complexity:

Three aspects of the multi-step processes involved in chemosensory acquisition can be clearly delineated in the dual olfactory systems [main (MO) and vomeronasal (VNO)] present in elephants.

a. Spatial: The unusual head anatomy of the elephant extends the main olfactory (MO) and VNO systems spatially and coupled with the huge size of these animals renders them unsurpassed for olfactory studies in the mammalian kingdom. Long truncal passageways (200 cm) precede vast enveloped turbinates that comprise 0.8 square metre of olfactory epithelial surface. Preceding the paired VNO (up to 20 cm long with an estimated epithelial surface of 18–38 cm^2) are ducts, 15–20 cm long (Johnson & Rasmussen, 2003) that can be aspirated using catheters to collect VNO mucus **(Fig.4)**. Both the large MO and VNO systems, with their abundant mucous secretions, have enabled the isolation, identification, and functional analysis of pheromone-binding proteins *in situ* as described above. Cloning and recombinant expression of the genes is required only for characterisation stages.

Figure 4. **The authors collecting VNO mucus from Kashin, an Asian elephant female from Auckland Zoo, by aspirating via a baby feeding catheter. [Don't try this at home].**

b. Temporal: In elephants, the vast size of this integrated chemosensory system effectively slows down the individual events in real time and ensures the ability to observe perireceptive events as discrete and quantifiable steps. This correlation allows corroborations of *in vitro* and *in vivo* measurements.

c. Interplay: The anatomical interrelationships between the MO and VNO systems are differentiated more clearly in elephants than other mammals. Anatomical and temporal interrelationships are reinforced by the role of truncal mucus in modifying the pH and sequestering released pheromone. The postulated VNO accessory duct into the trunk lumen provides a putative conduit for mucus exit via an hypothesised 'pump' for clearing and/or resetting the VNO. This helps support the observation that initial MO detection is followed by specific and quantitative assessment through the VNO by the flehmen response.

7. Behaviour correlates with biochemistry and physiology:

To a degree not demonstrable in other mammalian systems, each step of the biochemical studies we have performed — from working with the binding proteins and determining the kinetics of pheromone-protein interactions — can be confirmed by and coupled to bioassays using wild and captive elephants that reveal measurable responses and specific behaviours (Rasmussen & Greenwood, 2005). The elephant model VNO system can be used with individual animals repetitively to reveal details of VNO function, such as the importance of pH, the transport role of serum albumin, and the sequestering role of pheromone binding protein.

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NEWS

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The Elephant as an ideal olfactory model mammal

continued

Figure 5. A structural model of elephant serum albumin showing the position of several amino acid residues that can be photoaffinity labelled with a (Z)-7-dodecenyl acetate analogue at alkaline pH.

8. Evolutionary diversity:

The elephant model exhibits both specialisation and generalisation of ligand–protein interactions and their functional mechanisms in MO and VNO systems. Elephants, unlike rodents, do not have major urinary proteins (MUPs) in their urine, but instead use an alkaline-induced conformational change of albumin to bind pheromonal ligands (Lazar et al., 2004; **Fig 5)**. The pheromone binding protein in the trunk mucus on the other hand is a MUP homolog, but functions more as a ligand-sequestering agent than a transport protein (Lazar et al., 2002; **Fig.6**). Our finding of a liganding VNO ductal protein is now adding to this mechanistic information (D.R. Greenwood and L.E.L. Rasmussen, unpublished results). The recent discovery of the enantiomeric ratio requirement for bioactivity of frontalin provides us with an unique and outstanding pheromone system, similar to insects where the absolute configuration of molecules reaches a pinnacle of specificity in resultant behavioural responses (Greenwood et al., 2005a).

9. Multi-disciplinary study:

Perireceptive events in the elephant model can be examined from perspectives ranging from behaviour, anatomy and physiology through to biochemistry and molecular biology. Moreover, the genome sequence of the African elephant is now available and awaiting complete annotation. Homologous sequences in the Asian elephant reveal key similarities and differences that may help rationalise results from comparative semiochemical sensing experiments. Moreover, using molecular techniques, plentiful protein can be prepared for studying interaction kinetics, and future receptor function studies with defined ligands are anticipated.

10. Conservation keystone species and public science education:

Deciphering chemical communication used by Asian elephants is already helping resolve elephant–human conflicts in Southeast Asia (Rasmussen and Riddle, 2004). Moreover, human interest in such a charismatic species facilitates the promotion of scientific concepts and information to the general public with an empathy that is perhaps surprising. With the Asian elephant now considered to be on the brink of extinction, understanding and helping implement or enhance husbandry practices would seem a worthwhile undertaking in the light of the reduction in habitat and increasing incidence of human–elephant conflict situations.

Conclusion:

These criteria rival, if not surpass, those for model rodent species and lend credence to the notion that "size does matter" and that elephants, especially the Asian elephant, are indeed good olfactory models. Through careful and intensive analysis of elicited responses to compounds presented to wild and captive individuals, we have been able to define the role of several compounds present in various glandular secretions and excretions that initiate key behaviours in conspecifics that are important for maintaining social interactions and indeed, for ensuring the reproductive success of this endangered animal.

Acknowledgements:

We thank our many colleagues and collaborators over the years and especially Riddle's Elephant Sanctuary, The Ringling Centre for Elephant Conservation, Oregon Zoo and Auckland Zoo, where much of the sampling and testing has been undertaken. We acknowledge the following for funding our collaboration: Biospherics Research Corporation, ISAT and NSF [IOB#0544191 awarded to L.E.L.R and D.R.G but returned due to the death of L.E.L.R].

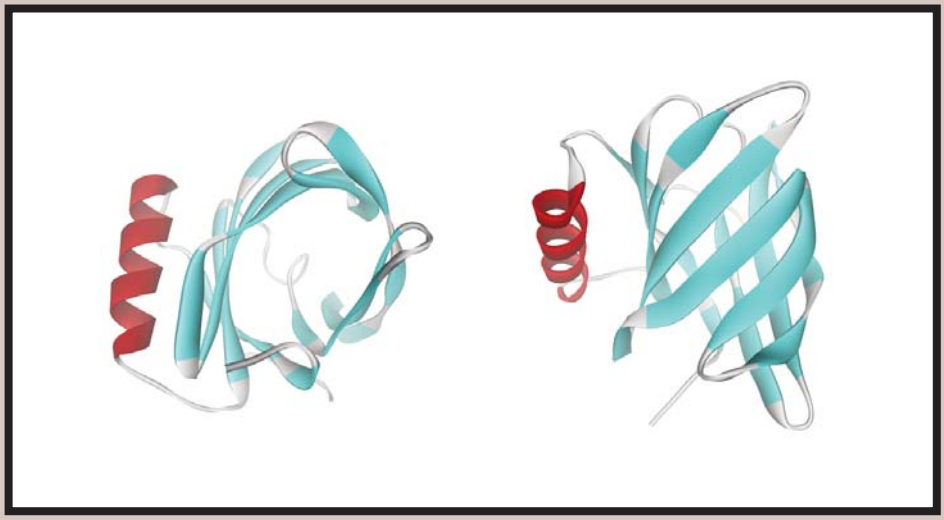

Figure 6. Two orientations of a structural model of the Asian elephant pheromone binding protein showing a hollow barrel with one end capped into which the pheromone (Z)-7-dodecenyl acetate fits.

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Prospecting for Mammalian Chemical Signals via Solventless Extraction Techniques: An Elephantine Task

Thomas E. Goodwin[1] and Bruce A. Schulte[2]

[1] Hendrix College, Department of Chemistry, goodwin@hendrix.edu
[2] Georgia Southern University, Department of Biology, bschulte@georgiasouthern.edu

INTRODUCTION

In contrast to a plethora of known insect pheromones, a paucity of mammalian pheromones has been identified, two of which have been in elephants (Albone, 1984; Brown and Macdonald, 1985; Wyatt, 2003; Burger, 2005). Elephants possess one of the world's best chemosensory systems, due in no small measure to their prehensile trunk. The trunk is not only the gateway to smelling (primary olfaction), but also the means by which chemical signals are conveyed from their source to the openings of the vomeronasal organ ducts in the roof of the mouth (the flehmen response; secondary olfaction) (Rasmussen, 1999). The late L. E. L. "Bets" Rasmussen was a pioneer in the study of chemical signaling among elephants (Goodwin and Schulte, 2007). Among her many notable accomplishments are the identification of the urinary, preovulatory pheromone of the Asian elephant (*Elephas maximus*; Rasmussen et al., 1996a), and a chemical signal of musth in Asian male elephant temporal gland secretion (TGS; Rasmussen and Greenwood, 2003).

When prospecting for elephant pheromones, or those from any other mammal, four tasks must be accomplished:

(1) *extraction* of the volatile organic chemicals from the biological matrix;

(2) *separation* of the chemical components of the extraction mixture from each other;

(3) *identification* of the chemical compounds present in the mixture; and

(4) *verification* of bioactivity of the putative pheromones.

In our elephant research, and in general with similar investigations, the workhorse methodologies for *separation* and *identification* are gas chromatography (GC) and mass spectrometry (MS), respectively. *Verification* is achieved through behavioral bioassays, which in the case of elephants are quantified using flehmens and other distinctive trunk behaviors (Schulte et al., 2005; Schulte, 2006.) In this article we will discuss extraction procedures, primarily solventless ones that have been employed in the search for mammalian chemical signals.

For several years, the authors, an organic chemist (T.E.G.) and an animal behaviorist (B.A.S.), reaped the benefits of a productive collaboration and friendship with Bets Rasmussen (a biochemist). The main focus of our team was a study of chemical communication among African elephants (*Loxodonta africana*) (for example, see: Schulte et al., 2004; Bagley et al., 2006; Goodwin et al., 2006; Loizi et al., 2009). Our chemical analyses of volatile organic compounds in elephant excretions and secretions have involved solventless, and thus environmentally friendly ("green"), extraction methodologies. This report is not intended to be a comprehensive review of such procedures and mammalian applications thereof, but rather will focus on our work, along with selected examples of the techniques employed and discoveries made by other researchers in this area. (For more detailed comparisons and discussions of solventless extractions, see the following: Baltussen et al., 2002; Pillonel et al., 2002; Bicchi et al., 2004.)

GREEN CHEMISTRY

Green chemistry has been defined as "the utilization of a set of principles that reduces or eliminates the use of hazardous substances in the design, manufacture, and application of chemical products" (Anastas and Warner, 1998; p. 11). Green chemistry is increasingly being taught and practiced not only in chemical industry (Constable et al., 2007), but also in chemical education (Anastas and Kirchhoff, 2002; Goodwin, 2004). As the fifth entry in their well-known Twelve Principles of Green Chemistry, Anastas and Warner (1998; p. 30) state the following: "The use of auxiliary substances (e.g., solvents, separation agents, etc.) should be made unnecessary wherever possible and innocuous when used." We were thus motivated to use various solventless sample pre-concentration techniques and subsequent analysis by GC-MS as a cornerstone of our chemical ecology research. In the sections below after a short discussion of traditional extractions with organic solvents, we highlight a

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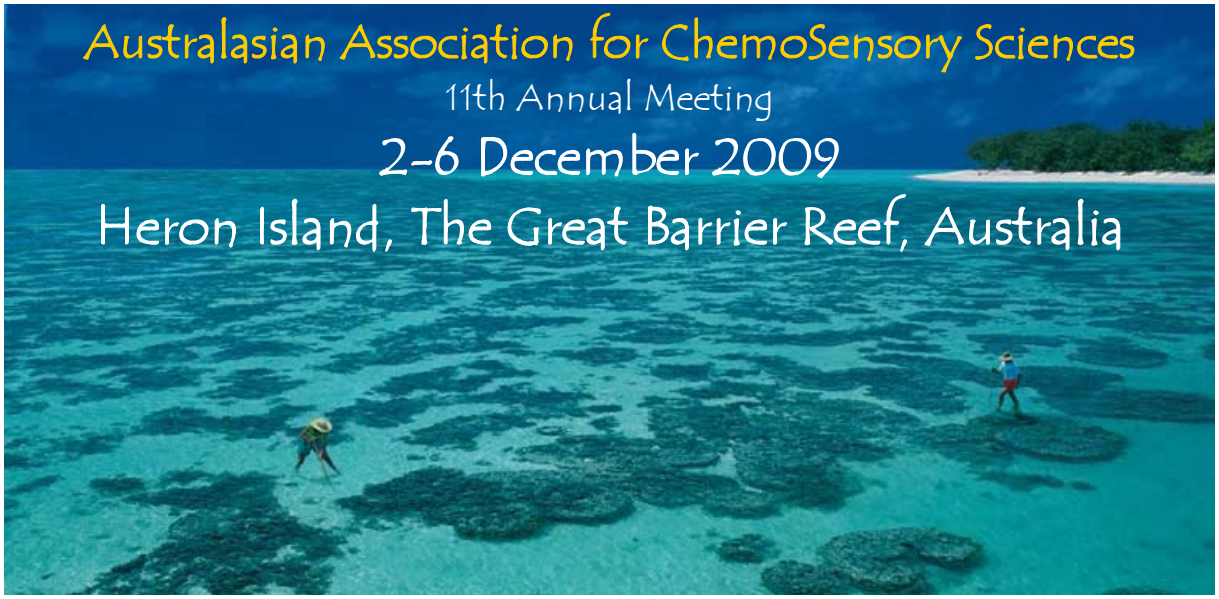

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continued

number of recent developments in solventless extraction of organic volatiles from aqueous solutions. For each technique, we present examples of the use of that technique for the study of chemical signaling in mammals.

SOLVENT EXTRACTION OF BIOLOGICAL SOLUTIONS

Traditionally, extraction of dissolved organic chemicals from dilute aqueous biological media has been carried out using a petrochemical organic solvent and a separatory funnel, or an apparatus for continuous solvent extraction. The extracts are then concentrated by solvent evaporation, the volatiles are cryoconcentrated (cryofocused), and analysis is by GC-MS (for example, see: Whittle et al., 2000; Zhang et al., 2003). Not only are these solvent-intensive procedures less environmentally benign, but there is also a danger of losing some of the more volatile analytes when the extract is concentrated via solvent evaporation. Nonetheless, solvent extraction coupled with bioassay-guided fractionation has led to some spectacular successes, notable among which is the 15 year quest by Bets Rasmussen to identify the pre-ovulatory pheromone in female Asian elephant urine. The specific estrous chemical signal, previously known as a sexual signal in many moth species, was found to be the simple ester Z-7-dodecen-1-yl acetate **(1)** (Z7-12Ac; Rasmussen et al., 1996a, 1997). Goodwin et al. (1999) also used this technique to analyze the temporal gland secretion (TGS) of African elephants, and identified several unusual sesquiterpenes, including (E)-2,3-dihydrofarnesol **(2)**, a bumblebee pheromone, and drimane-8 ,11-diol **(3)**, previously found only in a Greek tobacco.

SOLVENTLESS EXTRACTION TECHNIQUES USED WITH MAMMALIAN SAMPLES

TRAPPING ANALYTES ON A POROUS POLYMER

An early technique for solventless extraction of volatile chemicals from a sample's headspace (the vapor phase in equilibrium with a liquid mixture) involved passage of the vapor over a porous polymer (for example, Tenax®), followed by thermal desorption, cryoconcentration, and finally analysis by GC-MS. This process has proven to be very useful in the study of biological media (for example, see: Zlatkis et al., 1973; Jorgenson et al., 1978; Schwende et al., 1986; Service et al., 2001).

In recent years, several more powerful and simpler solventless methodologies have been developed, as discussed below.

EVACUATED CANISTER CAPTURE FOLLOWED BY CRYOGENIC TRAPPING (ECC/CT)

In a 1996 publication, Bets Rasmussen, her husband Rei, and their co-workers described how a novel technique named "evacuated canister capture-cryogenic trapping" (ECC/CT), originally developed for atmospheric sampling, could be adapted for the extraction of volatile organic chemicals from biological samples (Perrin et al., 1996; Rasmussen and Perrin, 1999). The technique involves the use of evacuated, scrupulously cleaned, inert, air-tight stainless steel canisters to collect sample headspace volatiles, pressurization of the canisters, and cryo-trapping the canister vapors in a liquid nitrogen-cooled U-tube containing 60-80 mesh glass beads, followed by gentle U-tube heating to release the analytes into the GC-MS. In this 1996 report, the TGS from a male Asian elephant was analyzed.

Among many subsequent applications of the ECC/CT methodology is a study of urinary chemical signals of musth in wild elephants in Kenya (Rasmussen and Wittemyer, 2002). Male elephants, both African and Asian, undergo a periodic rut-like condition called musth. Unlike rutting, however, musth is asynchronous among any group of male elephants. Characteristics of musth include elevated serum androgens (particularly testosterone and dihydrotestosterone), heavy drainage from the temporal glands, urine dribbling, increased aggressiveness, and enhanced success in competition for breeding (Eisenberg et al., 1971; Poole and Moss, 1981; Poole, 1987; Rasmussen et al., 1996b; Schulte and Rasmussen, 1999; Ganswindt et al., 2005). The Kenya study offers the first detailed musth/non-musth comparison of urinary chemical signals in both wild and captive African elephants. Not only were major differences observed between urinary volatiles from musth and non-musth samples, but also there were remarkable similarities to results obtained in earlier studies with captive African elephants, as well as with wild and captive Asian elephants. In particular, a group of ketones and alcohols was found to occur in greater amounts in musth versus non-musth urine in both species. These compounds are likely the result of increased metabolism of fatty acids during musth, and are thus chemical signals of musth to other male and female elephants.

ECC/CT also was used to analyze volatile organic compounds from three rather unusual elephant sources: (1) an aqueous solution that African (but not Asian) elephants eject from their ears (Riddle

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et al., 2000); (2) interdigital glands (Asian, but not African; Lamps et al., 2001; Rasmussen and Goodwin, unpublished); and (3) breath (Rasmussen, 1998; Rasmussen and Riddle, 2004). In addition, Bets used ECC/CT and a complementary solventless extraction technique, solid phase microextraction (SPME), to analyze female elephant urine and TGS of young musth males as discussed below.

SOLID PHASE MICROEXTRACTION (SPME)

SPME relies on a small glass fiber coated with an adsorbant polymer that can be exposed to the headspace over an aqueous sample, or immersed directly in the aqueous solution (Pawliszyn 1997; marketed by Supelco, Inc.). SPME has been employed for an immense variety of applications, due in part to its early development and ease of use (for example, see: Theodoridis et al., 2000). Selection of a particular SPME fiber (with various absorption polymer coatings) is based upon the properties of the analytes of interest. SPME is easily automated using the versatile and rugged Combi PAL GC autosampler (CTC Analytics). SPME is a static technique (the vapor does not move across the absorbant), unlike the older Tenax® dynamic methodology mentioned earlier. Additionally, the fiber is rather fragile and has a relatively low polymer loading.

Bets used the complementary techniques of ECC/CT and SPME plus GC-MS to identify and quantitate Z7-12Ac and other compounds in the pre-ovulatory urine of Asian elephants (Rasmussen, 2001). She and her collaborators also used ECC/CT and SPME to extract the sweet-smelling compounds that characterize musth secretions by young male Asian elephants (Rasmussen et al., 2002). Additionally, the beetle pheromone frontalin, having first been detected in male Asian elephant TGS using ECC/CT (Perrin et al., 1996), was demonstrated to be a chemical message of musth (Rasmussen and Greenwood, 2003). Even more remarkable was the demonstration that the nature of this chemical signal depends upon the enantiomeric ratio of frontalin in the TGS (Greenwood et al., 2005). In this latter project, SPME was used to extract frontalin from TGS.

We employed SPME/GC-MS to analyze African elephant TGS and found, inter alia, three 2,3-

dihydrofarnesol sesquiterpene derivatives **(4, 5, 6)** that were previously unknown to science (Goodwin et al., 2002). Structural assignments were confirmed by synthesis of these compounds from farnesol. We have not yet conclusively determined whether these sesquiterpenes and those reported in our earlier paper (Goodwin et al., 1999) serve as chemical signals among African elephants. In addition to our TGS analyses, we have used SPME to analyze female African elephant urine in the search for a preovulatory pheromone (Goodwin et al., 2005).

STIR BAR SORPTIVE EXTRACTION (SBSE)

Stir bar sorptive extraction (SBSE), a more recent development than SPME, offers some distinct advantages over the earlier technique (Baltussen et al., 1999; Baltussen et al., 2002; Kawaguchi et al., 2006). In SBSE, the absorbant polymer is coated on a small stir bar which is marketed by Gerstel, Inc. as Twister®. SBSE, originally developed for immersion use, has been adapted for headspace extraction in which the stationary stir bar is suspended above the liquid matrix. Implementation of SBSE is more expensive than SPME, requiring specialized add-ons for the GC-MS, but has a much thicker polymer coating and thus can extract a larger amount of analytes. SBSE has been used to extract a variety of organic compounds from human urine (Tienpont et al., 2002), and general treatises on the use of SBSE in a search for mammalian chemical signals have been published (Soini et al., 2005a; Novotny and Soini, 2007). In a seminal series of papers, the Novotny group and their collaborators have described a number of specific SBSE applications, including the following analyses: (1) hamster urine (Soini et al., 2005b); (2) ferret urine and anal gland secretion (Zhang et al., 2005); (3) mouse urine (Novotny et al., 2007); and (4) human axillary sweat, urine, and saliva (Penn et al., 2007). Additionally, these researchers have developed a "rolling stir bar sampling technique" in which the coated stir bar is fitted into a novel holder and rolled directly across the surface of interest (Soini et al., 2006). The analytes are then desorbed into a GC-MS. For example, extraction of surface volatiles from human skin, grapefruit, human fingerprints on a mirror, and bird feathers are described (Soini et al., 2007). A notable feature of this innovation is the ability to imbed an internal standard in the stir bar coating prior to sampling, thus allowing analyte quantitation.

SAMPLE ENRICHMENT PROBE (SEP)

Burger and co-workers developed a "sample enrichment probe" (SEP) for high-capacity extraction of analytes from gaseous and aqueous samples, followed by GC-MS analysis (Burger et al., 2006a). The probe itself consists of a thin rod of inert material (usually stainless steel), fitted on one end with a short sleeve of polydimethylsiloxane rubber tubing. SEP employs a much larger volume of sorptive phase than SPME, and yields results

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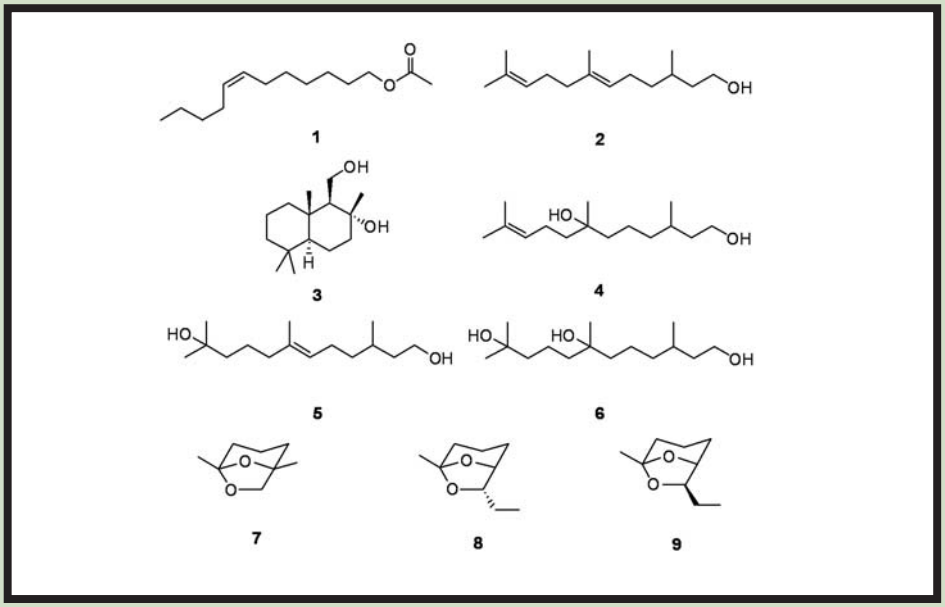
comparable to SBSE without the need for cryofocusing of analytes. Simple modifications to the GC inlet are required. This solventless extraction technique has been used successfully to analyze the urine of the cheetah, and the territorial marking liquid of the male Bengal tiger (Burger et al., 2006b, 2008). Two techniques that are similar to SEP are HCSP, an automated "high capacity sorption probe" (Pettersson et al., 2004), and SPACE ("solid-phase aroma concentrate extraction"; Ishikawa et al., 2004).

SOLID PHASE DYNAMIC EXTRACTION (SPDE)

Solid phase dynamic extraction (SPDE), the newest of the commercially available acronym trio that includes SPME and SBSE, features concentration of headspace analytes by repetitive flow back and forth over a polymer coating on the inside wall of a stainless steel syringe needle that is attached to a gas-tight syringe (Lipinski, 2001; marketed by Chromsys/Chromtech). SPDE has more absorbant polymer coating than SPME, but less than SBSE. SPME and SPDE have a larger variety of polymer coatings available than SBSE. SPDE is easily automated, as are SPME and SBSE. SPDE, unlike SPME and SBSE, is a *dynamic* technique for headspace analysis, and appears to offer some advantages for extraction of volatile organic compounds. The SPDE needle is more robust than the SPME fiber, has more extraction capacity, and for most applications can be used for hundreds of extractions before replacement. SPDE/GC-MS has proven to be useful in a variety of applications (see, for example, Musshoff et al., 2002; Bicchi et al., 2004; Ridgeway et al., 2006).

To our knowledge, we are the only group thus far to implement SPDE in the search for mammalian chemical signals. Most of our work has focused on a search for the putative preovulatory urinary pheromone in African elephants (Goodwin et al., 2007). Although male African elephants can distinguish conspecific female urine from different times in the estrous cycle (Bagley et al., 2006), specific chemical signals have not yet been verified. We employed SPDE/GC-MS to identify in female African elephant urine not only the beetle aggregation pheromones frontalin **(7)**, endo- **(8)** and exo-brevicomin **(9)**, but also their biochemical beetle precursors, thus suggesting a common beetle/elephant biosynthetic pathway (Goodwin et al., 2006). Extensive behavioral bioassays are underway to determine whether any of these compounds, or a blend of them, is functioning as a pheromone among African elephants. Most recently, we have begun to analyze volatiles in male African elephant urine to document not only musth/non-musth differences, but also how the mix of volatiles changes as young elephants mature, and as the urine ages after excretion. While elephant chemical signals remain our primary focus, our search for mammalian signals currently includes collaborative studies on lemur glandular secretions and urine, maned wolf urine, vole urine, putative interdigital glands of polar bears, and kakapo feather scents from a highly endangered, nocturnal, flightless parrot of New Zealand.



1 *= (Z)-7-dodecen-1-yl acetate,* ***2*** **=** ***2,3-****dihydrofarnesol,* ***3*** **=** *drimane-8,11-diol,* ***4*** **=** *2,3-dihydrofarnesol, 6,7-monohydrate,* ***5*** **=** *2,3-dihydrofarnesol, 10,11-monohydrate,* ***6*** **=** *2,3-dihydrofarnesol, dihydrate,* ***7*** **=** *frontalin,* ***8*** **=** *endo-brevicomin,* ***9*** **=** *exo-brevicomin.*

CONCLUSION

In this brief review, we have presented an overview of the various solventless extraction techniques that have been used to search for mammalian chemical signals. A major focus has been our own research on chemical signals in elephants, and the pioneering work of our friend and collaborator, the late Bets Rasmussen. Our search will continue to rely on chemical analyses in the laboratory, coupled with extensive behavioral bioassays with both captive and wild elephants. We hope that by learning more about these magnificent and endangered mammals, we can contribute to ensuring their long-term survival.

ACKNOWLEDGMENTS

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WineSense:

Geographical Indication and the Concept of Wine Typicality

Wendy V. Parr

Marlborough Wine Research Centre
Blenheim, New Zealand
wendyparr@xtra.co.nz

Geographical Indication

Great wines of the world are often known by place, Château d'Yquem and Domaine de la Romanée Conti being two that easily come to mind. In Europe, wine has been intimately tied to place for many years. New Zealand on the other hand is only just now in the process of legislating for geographical indication with respect to wine.

As with already established systems such as the appellation (AOC) system of Burgundy that was established in the late 19th and early 20th Centuries, the rules within New Zealand's geographical indication legislation are based on the importance of wine origin. Burgundy's AOC classification includes hierarchical considerations such as places of production considered "du bon terroir" (Jacquet, 2009) whereas New Zealand's focus is limited to designation of a wine as true-to-origin. The purposes of the geographical indication legislation include identification of, and ultimately protection of, any particular wine (e.g., in terms of marketing, branding, and legal aspects). It could also be argued that a further purpose is classification of wines in terms of distinguishing characteristics, such as a particular varietal expression within a particular production location (e.g., Marlborough's expression of Sauvignon blanc).

Concepts inherently related to geographical indication

Geographical indication is intimately tied to the concepts of *terroir* (see Jackson & Lombard (1993) or Moran (2001) for definitions) and to wine *typicality* (see Ballester et al., 2005 or Parr, Green, White & Sherlock, 2007). This is immediately evident when one considers that for legal purposes, geographical indications are defined as qualities and characteristics in the product that have their source "essentially attributable" to the geographic origin of the product (Barker, 2005). Although geographical indication is a political/legal creation (Barker, 2005), the locations distinguished may also be identifiable in terms of sensory phenomena associated with the products produced. That is, a delimited geographical area may produce a unique expression of a particular wine varietal in the sense that wines from such a location can be discriminated as different from other wines in a soundly-conducted scientific investigation.

The remainder of this article will focus on the relation between geographical indication and the concept of wine typicality.

Wine Typicality

Being produced within a delimited geographical area is not in itself sufficient to give a wine *typicality*. A wine with "typicality" is considered a wine that reflects both its geographical origin and varietal purity in a way that it forms a template or ideal against which other similar wines are measured (Charters & Pettigrew, 2007). In other words, typicality combines varietal expression (vins de cépage) and a particular location (vins de terroir).

Wine typicality is a cognitive or mental construct. It involves a representation in our mind (i.e., a memory), based on previous experience with wines and related products, as to what to expect in a good or ideal example of any particular product (e.g., a cheese or a wine). As we taste more Marlborough Sauvignons, for example, our concept of an ideal example will be constantly shifting in terms of both the central tendency (the ideal Marlborough Sauvignon) and the boundaries (e.g., with more experience, we may begin to include wines that were previously outside our concept such as those with subtle oak treatment). For obvious reasons, it has been argued that typicality can be assessed only by those with relative experience of the product under consideration (Sauvageot, 1994).

Inherently tying geographical indication to the typicality concept is the broad definition given 'source of origin'. In both concepts, source of origin includes history of a wine (e.g., classical variants), physical environment (e.g., soil type and other factors often summed up in the territorial and ecophysiological aspects of terroir (Moran, 2006), and cultural, social, and legal dimensions of the place of production (e.g., winemaking practices; socio-cultural mores of a community).

Current research concerning wine typicality

Over the last five years, a New Zealand-based programme of wine sensory research has been investigating wine typicality. Empirical studies, employing variants of established sensory evaluation methods, have investigated source of origin characteristics in Sauvignon blanc wines as perceived by participants with wine expertise (e.g., Parr, Frost, White, & Marfell, 2004; Parr, Green, White &

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Sherlock, 2007). The research employs an approach similar to that in a concurrent programme of research in France investigating typicality of Burgundy Chardonnay (e.g., Ballester et al., 2005). Questions addressed by the New Zealand research include: (i) What sensory characters are essential to define typicality of New Zealand's Sauvignon blanc wines from the Marlborough region? and (ii) Are wines from defined sub-regions within Marlborough distinctive in their perceived flavour profiles (e.g., Parr et al., 2004)? More recent studies have investigated the varietal expression of Sauvignon wines in a wider range of locations. For example, a recent study investigated the concept of Sauvignon typicality in relation to selected New Zealand, French and Austrian Sauvignons, and the flavour profiles associated with wines from the selected regions that were judged high in Sauvignon typicality (Parr et al., 2008).

How might the sensory research advantage the geographical indication legislation?

One goal of the empirical work described above is to provide scientific evidence, rather than rely on anecdotal reports, that wines from specific locations are distinguishable and/or identifiable on the basis of their perceived sensory characteristics. In other words, the aim is to identify places and wines where geographical distinctiveness and unique sensory phenomena come together. Such evidence, involving data from well-conducted sensory research, along with physical and chemical data, have potential to assist in providing one form of validation for the geographical indication concept.

An example of this is recent evidence in the form of sensory and chemical data that Sauvignon wines from one Marlborough sub-region, namely Awatere Valley, are distinctive in terms of both perceived flavour profile and chemical constituents. Across several studies, wines from the Awatere Valley have been judged the "most green" wines in flights of Marlborough Sauvignons from Wairau and Awatere locations (e.g., Parr, Sherlock, & Green, 2007; Parr, 2008). These sensory data are consistent with chemical analyses on the same wines that demonstrated higher levels of both isobutyl- and isopropylmethoxypyrazine in the wines from Awatere Valley, relative to wines from the other sub-regions. Wine producers in the geographically-identified area can then use the sensory data as they choose (for example, when modifying their viticultural operations or in their branding and marketing) if it appears advantageous to do so.

In conclusion, the legislation for geographical indication of wine is timely and necessary for New Zealand in both national and international contexts. It is suggested that sensory research investigating the closely related concept of wine typicality has potential to assist in validating the geographical indication concept by providing evidence that variants of any particular wine varietal, produced in a particular location, are perceived as distinctive relative to same-varietal wines from other locations. In identifying wines with typicality from delimited geographical areas, it will be important to appreciate that the typicality concept is nothing more than a mental representation and as such will be constantly changing in line with development of new variants of any particular wine style.

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A Reflective Tribute to L.E.L. "Bets" Rasmussen (1938-2006)

David R. Greenwood[1,2], Thomas E. Goodwin[3] and Bruce A. Schulte[4]

[1] New Zealand Institute for Plant and Food Research Ltd, Auckland, New Zealand
[2] University of Auckland, School of Biological Sciences, Auckland, New Zealand
[3] Hendrix College, Department of Chemistry, Conway, Arkansas, USA
[4] Georgia Southern University, Department of Biology, Statesboro, Georgia, USA

Diminutive in stature she may have been, but Professor L.E.L. Rasmussen ("Bets" to all who knew her) was a tower amongst mammalian chemical ecologists. Her pioneering work on olfaction in mammals, and elephants in particular, has laid the foundation for much of what we know about social chemicals and behaviour in large mammals. Through long hours of repetitive observations on wild and captive ani-mals her behaviour-directed bioassays were at the centre of her research approach, spending additional hours painstakingly pouring over video footage and enumerating the observed responses. At the other end, was the dedication to the fractionation and analysis of often evil-smelling secretions, exudates and excretions that formed the material for her testing regimes. Now almost 2.5 years after her untimely death, it seems an ideal opportunity to reflect back on her life and contribution as a person dedicated to science and humanity. We have already covered many of the salient features of her academic and practical contribution to the chemical ecology literature [Goodwin, T.E. and Schulte, B.A. (2007) A tribute to L.E.L. "Bets" Rasmussen (1938-2006). In: Chemical Signals in Vertebrates 11, J. Hurst, R. Beynon, T. Wyatt & C. Roberts (eds), pp. 1-9, Springer, New York], but at the time did not focus so much on Bets as a person and the underlying motivations for her life's work.

After raising her two sons, Bets began her studies on large, potentially dangerous and strange animals. She published on the endocrinology of lungfish, coelacanths and sharks before beginning her 15 year quest to identify the female-to-male sex pheromone of the Asian elephant. This work has spawned a great many other pro-jects, several involving the three of us. We have all benefited immensely from our association with Bets, deriving among other things a personal satisfaction from hav-ing collaborated with such a unique and determined individual, one who was totally dedicated to her mission to improve the lot for elephants, especially Asian elephants. These animals are now literally on the brink of extinction with fewer than 30,000 re-maining in the wild. She was dedicated to the cause of helping the Asian elephant survive as a species by minimizing human-elephant conflict with the help of local and overseas colleagues at zoos, sanctuaries and wildlife parks. Particular mention should be made of her friendship with Scott and Heidi Riddle, with whom she had a great bond and affection. This extended to working together on international conser-vation

Water skiing on the Waikato River (New Zealand). (Photo Dave Greenwood)

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Bets being filmed by Granada television collecting temporal gland volatiles from Riccardo. (Photo Dave Greenwood)

projects to develop, among other things, olfactory alternatives to electric fences to help reduce crop raiding (and hence human-elephant conflict) in India, as well as a 2002 Nature paper on the changing chemistry of musth in maturing Asian elephants. To the question of why study elephants Bets would reply "Have you ever seen an elephant? That's all the answer I need to give. I want to help preserve the elephant for future generations."

Bets possessed a phenomenal zest for life, still working on manuscripts while very ill in the months immediately before her death. She had an invigorating and affable personality with a zeal that extended into her bountiful writing. She delighted in us-ing and extending word usage and imagery. Her 'wordsmithery' was legendary, with the pheromone frontalin and its chiral 'reflections' capturing the essence of what she wanted to convey, somewhat akin to J.R.R. Tolkien's 'misty, moisty' evocative im-agery to which she aspired.

Bets and her husband, Reinhold (Rei), a well-renowned atmospheric chemist and recipient of the 2009 Outstanding Oregon Scientist Award, developed the SUMMA canisters for air (and breath) collection, deriving some commercial success as the Biospherics Research Corporation which helped support their respective research activities over many years. Bets quickly adapted these unique research tools to cap-ture volatile organic compounds emitted from a variety of mammals, and followed up with gas chromatographic separation and mass spectrometric identification back in the lab to produce many startling discoveries and subsequent publications.

D.R.G. first met Bets at the 1997 joint ISOT/AChemS conference in San Diego where we had nearby posters describing our respective latest pheromone studies. It was a planned meeting as I was then heading to Utah to begin a short sabbatical working with her collaborator Glenn Prestwich on the proteins responsible for binding Bets' beloved (Z)-7-dodecenyl acetate pheromone. We had the urine and trunk pro-teins provisionally identified within days; then came the big job of filling in the gaps. So began my fruitful association with Bets for many years, subsequently culminating in our joint NSF grant received less than two months before her health started fad-ing. There are many fond memories of working together, including filming for Na-tional Geographic, AACSS conferences on Heron Island, and spending productive hours in each other's laboratories. Perhaps the most memorable moment for me personally, however, was on a drive from Palmerston North to Havelock North in the North Island of New Zealand during her visit in early 2005 when I missed a crucial turn off, so intense were our discussions on the possible effects of chirality on ele-phant behaviour. Within days of her return home to Portland a chiral capillary GC column was under intensive use re-examining frontalin from hundreds of painstak-ingly collected and curated

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continued

temporal gland secretions dating back up to 20 years. Within a month we had the answer which culminated in our joint Nature paper pub-lished later that year. That unplanned 30 minute detour turned out to be a defining moment of our collaboration.

B.A.S. had a similar bonding experience in the early 1990's when he conducted postdoctoral research with Bets. As we were driving through the Midwest to a re-gional behaviour meeting, our discussions rambled from Asian to African elephants. The discussion finally came to a temporary break as I was famished and we stopped for gas and food. I realized then that Bets could talk, work and play endlessly with only a green pepper or can of tuna for nourishment. Yet, these intensive interactions set the stage for more than a decade of collaboration, including a collaborative US National Science Foundation-funded project with T.E.G. from which we are still benefiting today.

After a talk by T.E.G. in Wisconsin years ago in which our fledgling elephant re-search interests at Riddle's Elephant Sanctuary in Arkansas were mentioned, I was fortunate to be approached by a member of the Bets "fan club", and thus I learned of this intriguing "matriarch". That chance discovery led to a two-hour, get-acquainted Sunday afternoon Tom/Bets phone call, and the resulting years of friendship and research collaboration. Many wonderful memories of Bets stand out, high among which are the times we would work all day in her Oregon lab, and then head out to Eagles Crossing, the enchanting homestead that Bets and Rei established in the hills high above Portland. While overlooking the Columbia River valley from the Rasmussen aerie, we would enjoy far-ranging conversations as we munched on our dinners (including a delicious variety of Oregon berries) from before dusk until well after dark. Bets and Rei regaled this provincial lad with wild and wooly tales from their extensive travels, but the talk always turned sooner or later to elephants. We were research collaborators to be sure, but more importantly, we were friends.

Bets' intense love for water sports, and in particular her daily predawn swims and endurance water skiing, is renowned. At AChemS conferences held yearly in Sara-sota, Florida she would arise at 4:30 am, drive for several hours up to Lake Hamilton for a half hour skiing session, then back again in time for the day's full scientific pro-gramme. Then she would repeat the process the following day and often the day after that too. Rarely would she be ready to exit from the water after half an hour. Bets must have had a high pain threshold as she would water ski at home in Port-land on the Columbia River well into November, and was always proud to announce her first ski trip in each New Year sometimes as early as January.

Few people could keep up with Bets' intensity and resolve. She thrived on adven-ture and would endure the discomfort of being camped on an observation platform 10 m up a tree for a month in the wilds of India every night filming crop raiding by elephants, so as to document and understand the elephants' own intensity and re-solve. Perhaps the pinnacle of this adventurous approach to scientific endeavour must surely be the collection of breath from orcas, secretions emanating from the feet of polar bears, collection of urine from manatees and mucus from the vomero-nasal ducts of mature male elephants. This is how she undertook her life, living things to the max. With her passing has gone a true enthusiast and dedicated scien-tist, one from whom we all learned so much, and one who has left an enduring leg-acy of scholarship and incredible achievement. She is greatly missed ■

Bets Rasmussen and Dave Greenwood recovering aspirated VNO mucus collected from Kashin, a 32 year old female Asian elephant at Auckland Zoo. (Photo Martin Heffer)

Upcoming Events

22-26 April 2009	**AChemS XXXI Annual Meeting** Hyatt Sarasota, Florida, USA Contact: www.achems.org
28-30 April 2009	**EcoForum Conference and Exhibition** Australian Technology Park, Sydney Contact: www.ecoforum.net.au
19-25 July 2009	**Summer School on Human Olfaction** Dresden, Germany Registration deadline: 1 May 2009 Contact: thummel@mail.zih.tu-dresden.de Also: www.tu-dresden.de
6-9 September 2009	**19th CASANZ Conference** "Air Quality and Economic Development" Perth Convention Centre Perth, Western Australia Contact: www.iceaustralia.com/casanz2009
2-6 December 2009	**Australasian Association for ChemoSensory Science (AACSS)** Annual Scientific Meeting Heron Island, Great Barrier Reef, Australia Contact: j.stjohn@griffith.edu.au

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Advertising: Brian Crowley, crowbrin@hotmail.com
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