Chem. Senses 26: 611–623, 2001

Source and Cyclic Release Pattern of (*Z*)-7-Dodecenyl Acetate, the Pre-ovulatory Pheromone of the Female Asian Elephant

L.E.L. Rasmussen

Department of Biochemistry and Molecular Biology, Oregon Graduate Institute of Science and Technology, Beaverton, OR 97006-8921, USA

Correspondence to be sent to: L.E.L. Rasmussen, Department of Biochemistry and Molecular Biology, Oregon Graduate Institute of Science and Technology, 20000 N.W. Walker Road, Beaverton, OR 97006-8921, USA. e-mail: betsr@bmb.ogi.edu

Abstract

Female Asian elephants (*Elephas maximus*) release a pre-ovulatory urinary pheromone, (*Z*)-7-dodecenyl acetate (*Z*7–12:Ac), to signal males of their readiness to mate. *Z*7–12:Ac is quantitatively elevated during the follicular stage of estrus, reaching maximum concentrations just prior to ovulation, as demonstrated by two complementary headspace techniques: (i) evacuated canister capture followed by cryogenic trapping; (ii) solid phase microextraction (SPME), used prior to gas chromatography/mass spectrometry (GC/MS). These patterns were coincident with observed male behaviors and were consistent with biochemical and binding properties of the active ligand, including optimal binding pH. To release maximum amounts of *Z*7–12:Ac for quantitation, serum and urine samples from three mature female Asian elephants in their luteal and follicular stages of several estrous cycles were subjected to heat and pH changes and were then treated with protease prior to SPME–GC/MS analyses. When the post-luteal serum progesterone concentrations declined to baseline levels, *Z*7–12:Ac became detectable in the female urine. Throughout the follicular stage pheromone concentrations increased linearly with no apparent relationship to the two serum luteinizing hormone peaks. Pre-ovulatory urine also contained related compounds, including (*Z*)-7–12-dodecenol. The relative amount of this alcohol increased relative to acetate during long-term storage, with a proportional reduction in bioactivity. *Z*7–12:Ac was not detected in mucus samples from the urogenital tract. A potential precursor of *Z*7–12:Ac was identified in liver homogenates from female elephants in the follicular stage.

Introduction

The identification of (*Z*)-7-dodecenyl acetate (*Z*7–12:Ac) as the pre-ovulatory pheromone of the Asian elephant (*Elephas maximus*) offers an unusual opportunity to study the mechanisms of pheromonal cueing in mammals co-incidental with complex sexual behaviors (Rasmussen *et al.*, 1996, 1997). Although the Asian elephant has developed a sophisticated society with multi-generational transmission of information and a high degree of learned behaviors, the powerful behavioral message of impending female sexual readiness that elicits male sexual behaviors (including erections) is communicated by a single specific chemical. The identification of this compound presents opportunities for molecular studies of the origin of the signal by the female emitter and reception of the signal in the male responder. In the latter, reception is a multistep process which involves sequential transporter proteins and results in delivery of the active ligand (message) to the postulated chemoreceptive bipolar neurons of the vomeronasal organ (Rasmussen *et al.*, 1998, 1999; Lazar *et al.*, 2000). Well-defined bioresponses such as flehmens by male elephants demonstrate behaviorally the influence of the pheromone on reception. Such behavioral responses can be used to confirm the measured urinary content of *Z*7–12:Ac. However, to precisely elucidate the locations and originating tissue of *Z*7–12:Ac within the female elephant, resident tissues and release patterns of the pheromone were studied by concurrently using two gas chromatographic (GC)/mass spectrometric (MS) techniques to confirm its chemical identity and to measure its concentration.

Previous investigations established that *Z*7–12:Ac is present in the urine of female Asian elephants during the follicular period of estrus. Several consecutive measurements of one female elephant demonstrated that the concentration levels increased ~100-fold from the early follicular stage to the peri-ovulatory stage (Rasmussen *et al.*, 1997). Such increases were paralleled by male behavioral responses (flehmens) to expelled female urine, whether present on the urogenital orifice or legs, or on the ground, or to experimental samples (Rasmussen *et al.*, 1982, 1997, 1999). When the concentration of pheromone was sufficient, multiple flehmen responses and other pre-mating behaviors were observed (Rasmussen *et al.*, 1996).

This study poses three questions related to the biological source of the urinary pheromone within female Asian elephants.

First, what is the quantitative cyclical variation in the urinary concentration of *Z*7–12:Ac during the 13- to 18-week estrus period? Hormonal characteristics of the long estrous cycle of the Asian elephant include low serum estradiol levels during the lengthy follicular and luteal stages and high concentrations of serum progestins during the luteal stage (Hess *et al.*, 1983; Hodges, 1998). For the African elephant (*Loxodonta africana*) the major serum progestins are 5-α-pregnane-3,20-dione and 5-α-pregnan-3-ol-20-one (Heistermann *et al.*, 1997; Hodges *et al.*, 1997); for the Asian elephant, 5-α-pregnane-3,20-dione is the major serum progestin (Schwarzenberg *et al.*, 1997). Serum progesterone levels are about one-tenth of the level of the other progestins; however, as its assay is both accurate and inexpensive, progesterone is utilized to delineate the periodicity of the cycle. A rise in progesterone followed by a short dip and a greater sustained elevation indicates ovulation and the beginning of the luteal phase (Hess *et al.*, 1983). Progesterone levels subside prior to the follicular stage, which is characterized by two luteinizing hormone (LH) peaks, the first detectable 3 weeks before ovulation and the second just prior to ovulation (Brown *et al.*, 1991, 1999; Kapustin *et al.*, 1996). In addition to male response behavior, there are subtle but discernible quantitative changes in female behavior that occur during the estrous cycle; many are chemosensory based (Schulte and Rasmussen, 1999; Slade, 1999). Within these behavioral and hormonal parameters urinary concentrations of *Z*7–12:Ac were measured at weekly and/or daily intervals throughout estrus in three female elephants. In addition, as an auxiliary control, samples were analyzed from an unusual non-cycling female elephant. This female consistently attracted male elephants, exciting mating behaviors, including mounting. What were the temporal patterns and concentrations of *Z*7–12:Ac released by this female?

Two complementary headspace techniques, (i) evacuated canister capture followed by cryogenic trapping (ECC/CT) and (ii) solid phase microextraction (SPME) followed by GC/MS, were used to identify and quantitate *Z*7–12:Ac and other compounds.

Second, are additional related urinary compounds implicated in bioactivity? As a fascinating example of convergent evolution, *Z*7–12:Ac is not only the primary pre-ovulatory pheromone of female Asian elephants, but it is also a significant part of the pheromone blend of more than 140 species of insects. Among insects these pheromone blends are usually precise ratios of several, often related compounds. The question arises whether any of the many volatile compounds in pre-ovulatory elephant urine are related to *Z*7–12:Ac and whether any contribute to the bioactivity. These volatiles include several alcohols and aldehydes. Initial bioassays of the aldehyde dodecanal elicited low level responses from male elephants (0.17 ± 0.17 flehmen/h) (Rasmussen *et al.*, 1996); however, successive assays demonstrated responses diminishing to 0. Either dodecanal or dodecenal in combination with *Z*7–12:Ac did not increase or decrease the response levels of males (Rasmussen, 1998). Traces of the alcohol (*Z*)-7-dodecenol were observed in fresh pre-ovulatory urine, presumably from hydrolysis of acetate (Rasmussen, 1999; Rasmussen and Schulte, 1999). Tests of this compound by itself were negative; tested in combination with *Z*7–12:Ac neither enhanced nor diminished responses were detected (Rasmussen, 1998).

The stability and/or degradation of signals is an important aspect of chemocommunication. In the urinary samples used in this investigation we quantified compounds related to *Z*7–12:Ac, especially the hydrolysis product [the corresponding alcohol (*Z*)-7-dodecenol]. Of special interest was the relative proportion of (*Z*)-7-dodecenol and *Z*7–12:Ac in relation to storage times and temperatures.

Third, is the urinary pheromone *Z*7–12:Ac detectable in the circulatory system or urogenital mucus? The presence of the pheromone in urine suggests two possible sources: the secretory mucus along the lengthy (1 m) urogenital tract or the blood as an ultrafiltrate via the kidney. Saline washes of the urogenital tract of estrous females elicited bioresponses (albeit variable) by male elephants, suggesting the former (Rasmussen *et al.*, 1982). In addition, ultrasonic observations of the female urogenital tract shortly after the end of the luteal phase revealed accumulations of highly viscous mucus (Hermes *et al.*, 2000) (T. Hildebrandt and R. Hermes, personal communication). However, the consistency of male bioresponses to presented urinary samples, especially urinary samples just prior to ovulation, suggested that an ultrafiltrate via the kidney from the blood was also a reasonable source. To evaluate which of these tissues, urogenital tract mucus, blood and/or liver, could be the source or intermediate sites of *Z*7–12:Ac volatiles from these fluids or tissue homogenates were studied by the two GC/MS techniques.

Materials and methods

Sampling protocol

Urine and blood samples were obtained from three regularly cycling female Asian elephants and one acyclic female: Pet, age 44 years, at the Oregon Zoo (OZ), Portland, OR; Mary, age 26 years, at the Riddle Elephant Sanctuary, Greenbrier, AR; Shanti, age 22 years, at the National Zoo, Washington, DC; Rebe, age 27 years, at the Ringling Center for Elephant Conservation (RCEC), Polk City, FL. The latter elephant (Rebe) did not demonstrate cyclical elevations in serum progesterone, yet she consistently attracted male elephants and elicited flehmens and mating behaviors.

Blood samples were either immediately utilized for blood headspace volatile experiments or were processed as serum. Aliquots of the latter were frozen at –80°C until analysis for

steroid hormones by radioimmunoassay or by SPME prior to GC/MS.

Urine samples were either used fresh or aliquots were frozen at –80 or –20°C. Some urine samples from Rebe, Pet and Mary were analyzed in the fresh state. The majority of urine samples from Rebe were frozen immediately at –80°C. Most urinary samples from Pet were frozen immediately at –80°C; the remainder were frozen immediately at –20°C and subsequently (1 week later) at –80°C. Samples from Mary were frozen immediately at –20°C and subsequently (within 3 months) at –80°C. Samples from Shanti were frozen at –20°C for 2 years before analysis (Table 1). Mucus samples were obtained from Pet, Shine (age 16 years, OZ) and Minyak (age 34 years, RCEC) and immediately frozen at –80°C.

Hormone assays

Serum samples were analyzed by radioimmunoassay for progesterone (Hess *et al.*, 1983) or by SPME prior to GC/MS analysis to quantify *Z*7–12:Ac. Using the radioassay method of Hess *et al.* (1983), serum progesterone concentrations of the samples from Pet and Rebe were measured at the Oregon Regional Primate Research Center (Beaverton, OR); those from Mary were measured by Dr T.E. Goodwin at Hendrix College, Conway, AR. Samples from Shanti were analyzed for LH by Dr Janine Brown at the Conservation and Research Center, Front Royal, VA (Brown *et al.*, 1999). Ovulation was set as time 0 (T_0) and defined as the two-step rise in serum progesterone levels occurring near the time of the second LH peak (Brown, 2000). Time into estrus was defined as the number of weeks from T_0.

ECC/CT and GC/MS

Serum

Selected samples of whole blood were volatilized under controlled temperature and atmosphere conditions; these headspace volatiles were captured in evacuated stainless steel canisters prior to GC/MS analyses. Specifically, 25 ml of fresh whole blood was placed in a 500 ml clean glass jar fitted with a special lid that contained two Swagelok fittings. One fitting was connected to the jar via ultraclean Nupro SS-4H4 bellow-stem valves to a special stainless steel receiving bottle (0.85 or 6 l) evacuated to ~30″ Hg vacuum. Similar valves connected the other fitting to a source bottle of pure air pressured to 40 psig. Prior to starting the experiment pure air was flushed into the system. Next, the jar samples were heated to 37°C [elephant body temperature (Benedict, 1936)] and allowed to equilibrate for 30 min, thus allowing the development of headspace volatility. Subsequently, at 15 min intervals for 2 h the stainless steel evacuated receiving bottle was briefly opened to allow entry of the compounds developed in the headspace. At the end of the collection time the receiving canisters were pressurized with helium to 30 psig to ensure long-term storage (at room temperature) and to facilitate GC/MS analyses. Each experiment was run in duplicate. Further details are provided in Perrin *et al.* and Rasmussen and Perrin (Perrin *et al.*, 1996; Rasmussen and Perrin, 1999).

Urine

Urine samples were also prepared for GC/MS analysis by the ECC/CT method. Each ECC/CT sample was 100 ml of urine obtained from fresh urine flow directly into a clean glass jar fitted with the special lid. The apparatus was similar to that used for blood ECC/CT. Again, samples were heated to 37°C and allowed to equilibrate for 30 min, during which time the headspace volatiles developed. Then, at 30 min intervals for 2.5 h headspace compounds were drawn into the stainless steel evacuated receiving bottle. Again, receiving canisters were pressurized with helium to 30 psig to ensure long-term storage and facilitate chromatographic analysis.

Table 1 Freezing parameters and sample analyses of female Asian elephant specimens

Elephant	Age (years)	Fluid	Fresh	Frozen at –20°C	Frozen at –80°C	GC/MS analyses	
						ECC/CT	SPME
Mary	26	serum		×			×
Mary	26	urine		×	×	×	×
Pet	44	blood	×			×	
Pet	44	serum			×		×
Pet	44	urine	×	×	×	×	×
Pet	44	mucus			×		×
Rebe	27	blood	×			×	
Rebe	27	serum			×	×	×
Rebe	27	urine	×		×		×
Shanti	22	serum		×			×
Shanti	22	urine		×			×
Shine	16	mucus			×		×
Minyak	34	mucus			×		×

The sample introduction system for subsequent GC/MS analyses of serum or urine headspace volatiles contained within these pressurized stainless steel canisters involved initial release of the volatiles from the canister and their sorption onto an in-line tenax trap. In turn, desorption from the tenax, employing a six port valve in line with a U tube cryogenic trap (0.125 o.d. × 9″) containing 60/80 mesh glass beads, was followed by cryogenic focusing on this loop. Compounds were then released from the loop by heat and separated and identified by GC/MS.

GC/MS analyses

The analyses were conducted on a Hewlett Packard 5890A gas chromatograph and a Hewlett Packard 5970B mass spectrometer. The gas chromatograph used a DB-1 (0.25 mm i.d. × 60 m × 1.0 μm film thickness) polymethyl silicone-coated capillary column (J & W Scientific). The gas chromatograph oven was temperature programmed from –60 to 200°C at 4°C/min, with a 5 min hold at an initial temperature of –60°C. The mass spectrometer was programmed for a mass scan of 33–300, which allowed for identification of compounds from C3 through C14. The conditions allowed quantitation as low as 0.10 ppbv. Compounds were identified using an NBS 75 K Hewlett Packard Mass Spectrometer Chem Station library search and were manually rechecked with the NIST/EPA/NIH Mass Spectral Data Base v.4.01. Internal standards of authentic compounds of measured concentration were employed for compounds of interest.

Solid phase microextraction (SPME)

Serum

Aliquots (500 μl) of selected serum and urine samples through the estrous cycle of Pet and paired serum and urine samples from Rebe were analyzed for *Z*7–12:Ac by SPME prior to GC/MS. The vials used for SPME were steam cleaned with hot distilled water, rinsed three times with triple distilled water and air dried prior to addition of urine. The top of the vial had a reverse direction insert that had been conditioned for several days in a gas chromatograph oven at 250°C. Based on initial results from urine analyses after SPME, 100 μm polydimethylsiloxane (PDMS) fibers were used. Immersed and non-immersed procedures were tried. A small cleaned magnetic stirring bar was added to each SPME vial. For each sample the adsorption of compounds/volatiles to the fiber was conducted first at ambient temperature (25°C) and then heated to 37°C. For selected duplicate samples the pH was adjusted to the optimal binding pH for a recently characterized protein that binds the active ligand, *Z*7–12:Ac (Lazar *et al.*, 2000). To other duplicate samples 1 mg/ml of non-specific bacterial protease (Sigma) was added (Poon *et al.*, 1999; Yamazaki *et al.*, 1999). Sorption times varied between 15 min and 1 h. At the end of the sorption time the fiber was retracted into the protective needle. The SPME needle was immediately inserted into the injector port of the gas chromatograph fitted with a special glass liner insert and the fiber was exposed for 10 min to allow desorption at 250°C, the temperature of the injector port. Thus, compounds adsorbed on the PDMS fiber were desorbed and focused onto the beginning of the gas chromatograph column (Arthur and Pawliszyn, 1990).

Standards and blanks

Because of the tendency of *Z*7–12:Ac to adhere to glass or plastic surfaces (Prestwich, 1987), between three and five blank samples were run prior to analysis of standards and samples. Separate SPME fibers and inlet liners were used for standards and samples. In addition, fibers were always reconditioned twice between analyses, with a blank analysis conducted after the second conditioning. After samples containing high concentrations (μg or greater) of *Z*7–12:Ac were analyzed the fibers were desorbed during five blank runs before additional samples were analyzed. These blank runs also ensured that no ghosting occurred on the gas chromatograph column itself. The glass liner was also cleaned and replaced after standards or samples with high concentrations of *Z*7–12:Ac had been run.

In wild and captive bioassay situations the urinary pheromone signal is biologically viable for 24–48 h (Rasmussen and Schulte, 1999; Rasmussen and Krishnamurthy, 2000). In an experiment designed to assess the stability of *Z*7–12:Ac under environmental conditions two separate 1 μl aliquots of *Z*7–12:Ac in 10 μl of distilled water were maintained and assessed for 24 h, one at 20°C (room temperature) and the other at 35°C (Asian environmental temperature). The sample maintained at 20°C was also assessed at 48 h.

Urine

For urine samples the SPME procedure was similar, but not identical, to that for serum. The vials were cleaned in a similar fashion. Urine samples were either fresh or thawed from aliquots previously frozen at either –80 or –20°C; storage time and temperature were recorded. Urine (1 ml) was placed in special 2 ml vials for SPME. In addition, for a number of urine samples 10 μl aliquots were carefully pipetted with a microsyringe into the bottom of 50 μl microcapillary tubes. For both scenarios the SPME fibers were exposed in the headspace above the liquid sample.

Optimal extraction (i.e. maximum absorption or adsorption to SPME fiber) was assessed by altering several conditions. First, several fiber substrates, including 100 μM polydimethyl siloxane (DMDS), 7 μM DMDS, 30 μM DMDS and 50/30 μM divinyl benzene/carboxen on DMDS on a Stableflex fiber, were tested for maximal quantitation of *Z*7–12:Ac. Results from the DMDS fiber were consistent and the method was standardized using this fiber. Total absorption was significantly increased by the use of a small magnetic stirring bar in each 2 ml vessel. Such stirring was not possible in microcapillary tubes. Initial sorption trials

were conducted at room temperature (25°C). Subsequently, the temperature of the vial was increased to 37°C to effect vaporization of more molecules of *Z*7–12:Ac. Next, the pH was lowered below the optimal binding pH of albumin, the primary urinary transporter (Lazar *et al.*, 2000), thus encouraging the release of *Z*7–12:Ac.

In a series of experiments 1 mg/ml of non-specific bacterial protease (Sigma) was added to heated, stirred but non-pH-altered urine to additionally release *Z*7–12:Ac from its urinary albumin carrier. Volatiles were adsorbed for periods of 15 min to 1 h during most experiments, using the first four parameters. After sorption the fiber was retracted and the needle inserted into the injector port of the gas chromatograph fitted with a special gas liner insert. The temperature of the injector port was 250°C. Compounds adsorbed on SPME fibers were desorbed prior to GC/MS.

One experiment was conducted to simulate the release rate in the natural situation and to determine the total amount of *Z*7–12:Ac in 1 ml of follicular urine. Exhaustive extraction of a single 1 ml aliquot of urine was carried out over a 3 day period in three 12 h sessions. Between the 12 h sessions the sample was frozen at –80°C. Two separate PDMS fibers were alternately used to adsorb so that one fiber was being analyzed while the other was being adsorbed. In the first session, during the first 3 h, the sample was heated to 37°C, then the pH was lowered to 6 and similar sampling/analyses were conducted for the next 3 h. During the final 6 h, 1 mg of protease was added and the temperature was maintained at 37°C. For sessions 2 and 3 the temperature was maintained at 37°C.

Mucus and liver homogenates

Aliquots (1 ml) of cervical and vaginal mucus samples were heated to 37°C in 2 ml SPME vials. Volatiles were allowed to sorb onto a PDMS fiber for 30 min. Desorption in the inlet port of the GC was for 10 min at 250°C.

Liver tissue from a pre-ovulatory female stored frozen at –80°C was homogenized 1:1 in buffered saline, pH 7.5. Homogenate (1 ml) was placed in SPME vials and heated to 37°C. The PDMS fiber was exposed to the volatiles above the warmed homogenate for 15 min. The fiber was desorbed for 5 min at 250°C in the injector port of the gas chromatograph.

GC/MS analyses

GC/MS analyses for the SPME experiments were conducted using a Hewlett Packard 6890A gas chromatograph and a Hewlett Packard 5973 mass selective detector. The gas chromatograph column was identical to that used for ECC/CT, i.e. a DB-1 0.25 mm i.d. × 60 m × 1.0 μm film thickness polymethyl silicone-coated capillary column (J & W Scientific). The gas chromatograph oven was temperature programmed from a 4 min hold at 40°C, to 200°C at 6°C/min, then at 2°C/min to a final temperature of 235°C.

The mass spectrometer was programmed at 0.83 scans/s for a mass scan of 33–550, which allowed identification of compounds from C3 through C18. Most compounds were identified using an NBS 75 K Hewlett Packard Mass Spectrometer Chem Station library search and were manually rechecked with the NIST/EPA/NIH Mass Spectral Data Base v.4.01+1.6d and the Wiley Library v.6-275. For *Z*7–12:Ac and (*Z*)-7-dodecenol authentic synthetic standards were analyzed on our particular equipment, allowing confirmation of two parameters of identification: retention time by GC separation, and mass ion and dominant ion patterns by mass spectroscopy (Figure 1A and B). Especially for the samples analyzed by ECC/CT–GC/MS, peak areas of internal authentic standards, compared with

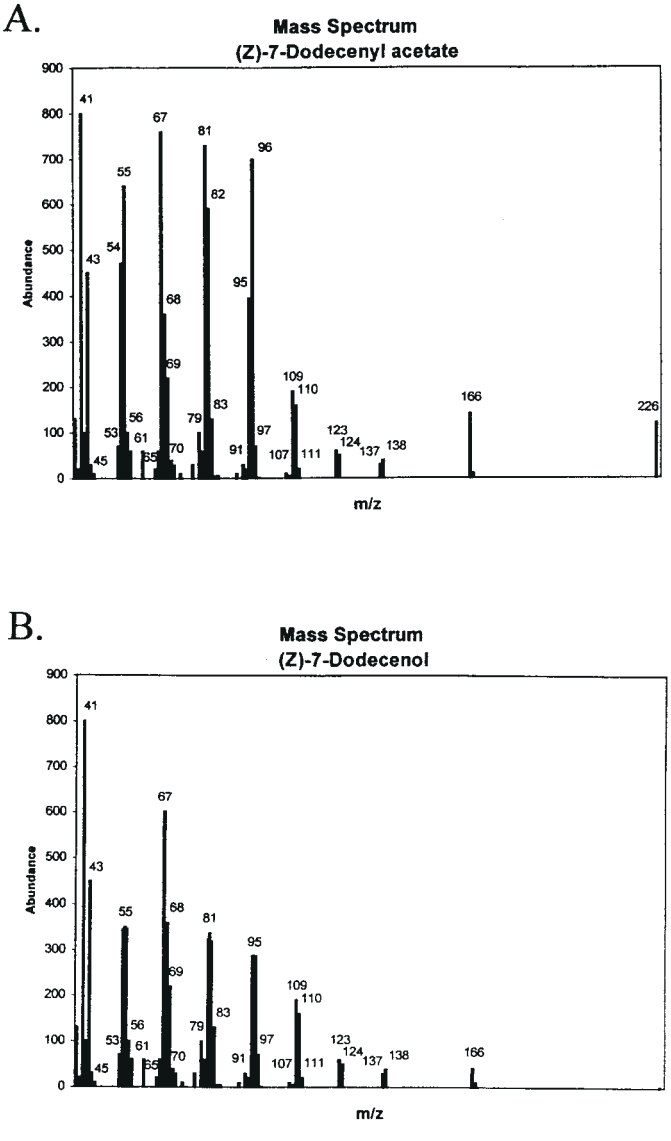

Figure 1 Mass spectra of **(A)** *Z*7–12:Ac and **(B)** (*Z*)-7-dodecenol.

real samples, and the use of TO12 standards allowed quantitation of the compounds of primary interest.

Results

Question 1: what is the quantitative cyclical variation in the urinary concentration of *Z*7–12:Ac during the 13- to 18-week estrus period?

Urinary pH and protein concentrations

Biochemical constituents in the urine and serum relevant to the pheromone *Z*7–12:Ac were quantitatively different during the follicular and luteal stages. Urine pH and protein concentrations were significantly different during specific stages of the estrous cycle. pH measurements of fresh urine demonstrated that pH was significantly elevated during the follicular stage compared with the luteal stage (Table 2). In addition, total protein content was elevated during the follicular stage compared with the luteal stage, despite a constant creatinine concentration (Table 2).

Concentration of Z7–12:Ac

Initial measurements by ECC/CT–GC/MS. Initially *Z*7–12:Ac was detected by ECC/CT–GC/MS (Rasmussen *et al.*, 1997). Identification was possible by the characteristic mass spectrum pattern for this compound (Figure 1A). Quantitation was difficult, because the pheromone presumably eluted unevenly from the tenax and may have adsorbed to the glass beads in the cryogenic loop. The initial measurements were possible at the pre-ovulatory stage, but detection at other times during the follicular stage was difficult. Therefore, ECC/CT–GC/MS was utilized primarily to identify the dominant volatile compounds, especially those related to *Z*7–12:Ac (Table 3). SPME followed by GC/MS was used for initial and subsequent quantitation of *Z*7–12:Ac (Tables 2 and 4).

Determination of optimal extraction conditions by SPME–GC/MS. Using the 1 ml specimens of pre-ovulatory urine conditions for optimal volatilization and maximal extraction of *Z*7–12:Ac were assessed. Factors considered included the ratio of headspace volume to liquid sample, temperature, mixing vortex and pH. In addition, as Z7–12:Ac binds to elephant albumin (Lazar *et al.*, 2000), the effect of a non-specific protease was also recorded (Table 4). Increasing the temperature from 25 to 37°C almost doubled the concentration of *Z*7–12:Ac detected (Table 4). Lowering the pH increased release of the pheromone; measured concentrations increased another 4-fold. Finally, addition of protease resulted in an additional 10-fold concentration (Table 4 and Figure 2).

Exhaustive extraction of Z7–12:Ac (1 ml). The systematic exhaustive extraction of 1 ml of late pre-ovulatory urine involving 36 SPME–GC/MS analyses during a 3 day period yielded a total of 20 μg of *Z*7–12:Ac.

Urinary concentrations of Z7–12:Ac during estrus (1 ml and 10 μl). Using the SPME scheme indicated by the preceding results the concentrations of *Z*7–12:Ac were measured in fresh urine samples from Pet (female 1) and in frozen samples from Mary (female 2) and Shanti (female 4) (Table 5). The fresh urine samples from Pet delineated the linear increase in pheromonal concentrations as the female approached ovulation (Table 5). Interestingly, one sample from the hormone transitional period between the late luteal and very early follicular stages contained very high (1.0 mM) levels of *Z*7–12:Ac. Explanations are suggested in the Discussion. In addition, urine from the non-cycling female (Rebe), which consistently attracted male elephants, contained consistently high concentrations of *Z*7–12:Ac (Table 5).

When *Z*7–12:Ac concentrations were measured relative to serum LH peaks in urine samples from Shanti (female 4), several relationships emerged (Table 5). *Z*7–12:Ac concentrations (0.020 mM), measured on the same day as the first LH elevation, were not increased. However, five daily measurements of *Z*7–12:Ac (SDS-1–5), concurrent with serum LH assessments around the time of the second LH peak, demonstrated an interesting elevation pattern (Figure 3). *Z*7–12:Ac (SDS-3) was elevated prior to the 20 ng/ml LH serum peak. Subsequently, *Z*7–12:Ac concentration began

Table 2 Urinary constituents relevant to the pheromone during the estrous cycle

Estrous stage	Urinary pH[a]	Serum P_4 (pg/ml)	Urinary proteins (μg/ml)[b]		*Z*7–12:Ac (mM)[c]
			Range	Mean ± SE	
Luteal	7.67	400	56–74	64.7 ± 1.91	not detected
Early follicular		175	54–65	57.8 ± 1.55	0.002
Mid-follicular		76	78–90	84.2 ± 1.39	0.058
Pre-ovulatory	8.34	35	90–104	97.6 ± 1.45	0.146

[a]Mean urinary pH ($T = 2.5$, DF = 8, $P = 0.037$).

[b]Creatinine varied from 75 to 100 ng/ml, with no significant difference between stages.

[c]From Rasmussen *et al.* (Rasmussen *et al.*, 1997).

Table 3 Some dominant and relevant urinary compounds as detected by ECC/CT and SPME GC/MS

Compound	Relative retention time (min)		Bioactivity (flehmens/h)
	ECC/CT	SPME	
Acetaldehyde	18.30		na
Pentanal	42.80		na
4-Methyl-3-penten-2-one	48.80	11.84	sip
4-Heptanone	53.10		sip
4-Methylphenol	63.00	23.71	na
Acetophenone	63.30		na
Decanal	69.60	26.67	na
2-Buten-1-one 4-(2,6,6-trimethyl)cyclohexen-y-yl	67.44	30.57	
2-Oxabicyclo[4.4.0]dec-7-ene	ni	30.92	sip
2,6,10,10-Tetramethyl-1-oxa-spiro[4.5]dec-6-ene	ni	31.22	sip
3-Dodecen-1-al	ni	31.85	nt
4′-Methoxyacetophenone	75.10	32.80	sip
1,1-Dodecanediol acetate	76.00	32.90	na
2-Dodecanone	78.00	32.90	0.17 ± 0.17[a]
Dodecanal	79.00	33.00	na
2-Dodecanol	80.00	34.40	na
(*Z*)-7-Dodecenol	89.00	34.50	na
3-Buten-2-one 4-(2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1-yl)[b]	nr	35.45	nt
(*Z*)-7-Dodecenyl acetate	96.20	38.25	active
Cyclodecanone	nr	40.05	nd
Dodecalactone	nr	40.76	nd
Methyl ester of hexadecanoic acid (*N*-hexadeconoate)[b]	nr	50.10	nt

na, no bioactivity detected during bioassay; ni, not identified; nr, not resolved; nt, not tested; sip, study in progress. Cyclododecanone, 1,8-dodecadien-1-yl acetate and 8,10-dodecadien-1-ol bioassayed negative.

[a]$n = 16$.

[b]Liver homogenate.

Table 4 Measurement of *Z*7–12:Ac concentration: pre-ovulatory urine (urine/headpace ratio 1:1)

Condition	1 ml samples (mM)[a]	10 µl samples (mM)[a]
25°C (pH 8.0)	0.014 ± 0.003	0.009 ± 0.002
37°C (pH 8.0)	0.020 ± 0.002	0.018 ± 0.003
pH 6 (37°C)	0.095 ± 0.014	0.086 ± 0.009
Protease-treated (1 mg/ml)	0.873 ± 0.284	0.079 ± 0.098

[a]Mean ± SE.

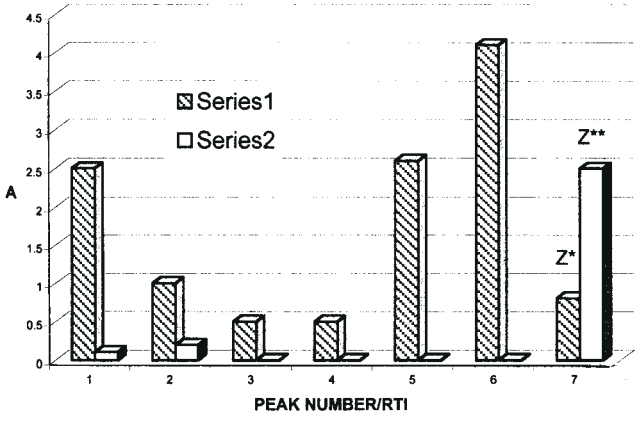

Figure 2 SPME–GC/MS analyses of *Z*7–12:Ac concentrations in pre-ovulatory late follicular stage urine. Peak 7 was identified as *Z*7–12:Ac by mass spectra analyses. This peak is depicted graphically and quantitatively. In series 1 the urine was heated to 37°C. Z*, concentration of *Z*7–12:Ac was 0.02 mM. In series 2 the urine was also heated to 37°C. In addition, protease was added. Z**, concentration of *Z*7–12:Ac was 0.151 mM. A, abundance; RTI, retention time interval.

decreasing; within 49 h after ovulation concentrations were non-detectable, which is characteristic of the luteal stage (Table 5).

Question 2: are compounds related to *Z*7–12:Ac present in urine and do they contribute to or affect bioactivity?

Acetate:alcohol ratio changes with long-term storage

Under the appropriate pH and temperature conditions and after the addition of protease the dominant compound resolved in fresh pre-ovulatory urine was *Z*7–12:Ac; only trace amounts of the corresponding alcohol, (*Z*)-7-dodecenol, were measured (Table 6, footnote c). However, urine frozen at –20°C for long periods demonstrated a diminished percentage of acetate and an increased percentage of the

Table 5 Urinary concentrations of pheromone during the estrous cycle (SPME–GC/MS) measurements

Estrous state	Female no.	Serum P_4 (pg/ml)	Serum LH (ng/ml)	Urine *Z*7–12:Ac protease-treated (mM)
Luteal (weeks 1–6)	1, 2	150–800	0.27–0.45	not detected
Early follicular (weeks 8–10)	1	<100	0.45–0.73	0.002 ± 0.0003
	2	<100		0.012 ± 0.001
	3 (one sample)			1.0
Mid follicular (weeks 11–14)	1	<50		0.085 ± 0.002
	2			0.019 ± 0.001
	4 (at first LH peak)		3.66	0.020
Late follicular (weeks 15–16)	1	<25		0.873 ± 0.284
	2			0.476 ± 0.133
SDS-1	4		0.42	0.212
SDS-2	4		0.39	0.440
SDS-3	4		0.42	0.890
SDS-4	4 (at second LH peak)		20.00	0.530
SDS-5	4		0.91	0.190
Non-cycling (attractive to male elephants)	5	<25		0.14–0.95

1, Pet (mean of three SPME–GC/MS measurements); 2, Mary (mean of three SPME–GC/MS measurements); 3, Shine (1 sample); 4, Shanti; 5, Rebe (range of five samples). LH, luteinizing hormone; SDS, successive daily samples 1–5.

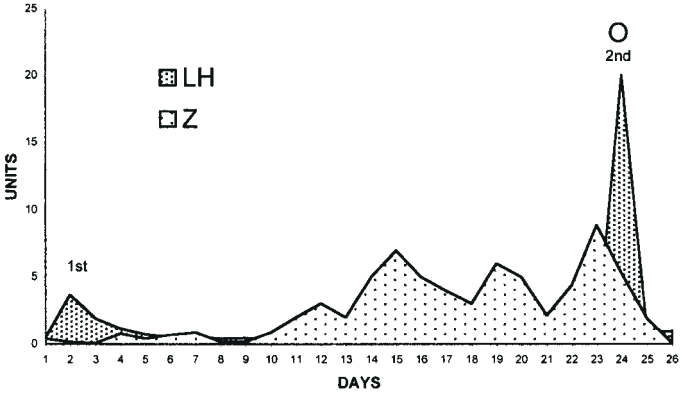

Figure 3 Luteinizing hormone (LH), *Z*7–12:Ac and progesterone were measured during the 24 days prior to ovulation (O), at the day of ovulation and the day after. Progesterone levels were <25 pg/ml until ovulation. First (1st) and second (2nd) LH peaks are indicated. Elephant Shanti. Units for LH, ng/ml; units for *Z*7–12:Ac, 1×10^{-1} mM.

alcohol. As storage time increased the percentage of alcohol increased, suggesting a gradual hydrolysis at this temperature (Table 6, lower left). In contrast, urine stored at –80°C did not demonstrate elevated alcohol proportions over time.

Proportionality changes in pure Z7–12:Ac

The maintenance of *Z*7–12:Ac in distilled water at two temperatures, 20°C (room temperature) for 24 and 48 h and at 37°C for 24 h, demonstrated a gradual hydrolysis to the corresponding alcohol (Table 6, top and middle).

Relationship of bioactivity to the acetate:alcohol ratio

Bioresponses by male elephants declined as the total and proportional concentration of *Z*7–12:Ac in a bioactive chromatographic fraction declined with storage time. Over a 10 year storage period the proportion of *Z*7–12:Ac diminished by 25%. In a 5 year period the bioresponses declined from 5.2 to 3.9 flehmen/h, a 25% decline (Table 6, lower right).

Table 6 Ratio alterations of acetate to alcohol: effect on bioactivity

	*Z*7–12:Ac:alcohol ratio:		Male responses[b] (flehmen/h) (n = 3)
	in urine	in water[a]	
Initial time		Pure	
Time at temperature			
24 h at 20°C		1000:1	
48 h at 20°C		750:1	
24 h at 40°C		500:1	
Time in –20°C freezer			
Initial time (fresh)	100:0[c]		5.2
24 h	99:1[d]		
1 week	98:2		
1 year	95:5		
5 years	85:15		3.9
10 years	75:25		

[a]Ultrapure (*Z*)-7-dodecenyl acetate (Pherobank, The Netherlands).

[b]Bioresponses to *Z*7–12:Ac-rich flash chromatographic fraction. This HPLC fraction also contained 1,1-dodecanediol acetate and dodecalactone.

[c]At times traces of alcohol were detected.

[d]At room temperature (20°C) the ratio was 98:2.

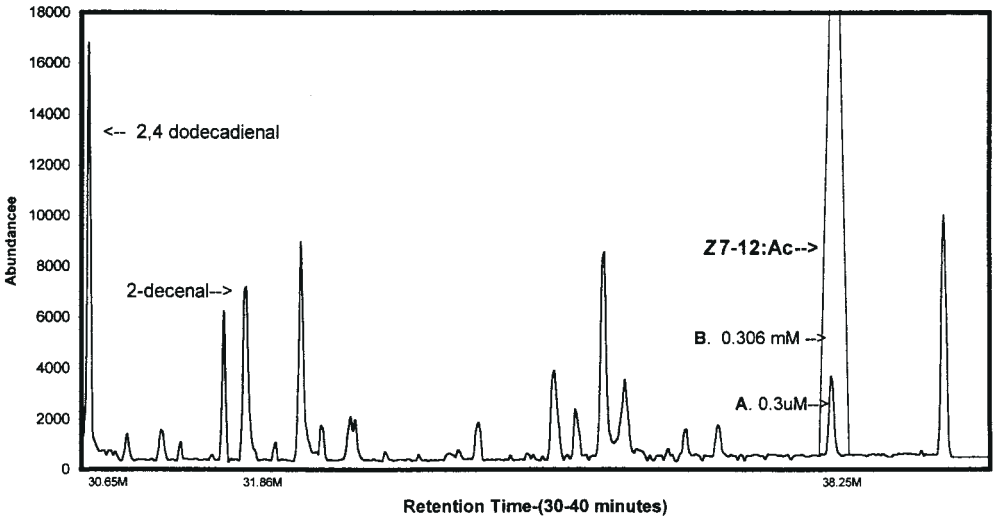

Figure 4 SPME–GC/MS analyses of *Z*7–12:Ac concentrations of **(A)** native preovulatory serum heated to 37°C and **(B)** the same serum aliquot with addition of protease. The *Z*7–12:Ac peak is indicated in both **(A)** and **(B)**.

Presence and bioactivity of related compounds

In fresh pre-ovulatory urine samples, in addition to (*Z*)-7-dodecenol, other related compounds have been identified (Table 3). 2-Dodecanone, (*Z*)-7-dodecenol, dodecanal, 1,1-dodecanediol acetate and dodecalactone have not been demonstrated to be individually biologically active (i.e. to elicit flehmen responses and pre-mating behaviors from male Asian elephants) nor do they enhance or diminish bioactivity in combination with *Z*7–12:Ac.

Question 3: is the urinary pheromone *Z*7–12:Ac detectable in the circulatory system or urogenital mucus?

Serum

Serum from two female elephants in their pre-ovulatory periods (Pet and Shanti) and the non-cycling, attractive female (Rebe) contained measurable amounts of *Z*7–12:Ac. *Z*7–12:Ac concentrations up to 0.3 μM were assessed in native serum when it was heated to 37°C (Figure 4). When the serum was treated with protease at 37°C and the pH was reduced to <7, 0.306 ± 0.1 mM *Z*7–12:Ac was measured in serum (Figure 4).

Mucus

In contrast, urine-free samples of mucus, some obtained directly from the 1 m long female reproductive tract, contained no detectable *Z*7–12:Ac. The absence of 4-methylphenol, the dominant volatile organic in urine, confirmed the integrity of the mucus sample. The primary compounds, as assessed by SPME followed by GC/MS, included acetic acid, propionic acid, dodecanoic acid, benzoic acid and isopropyl myristate. The implications of the presence of dodecanoic acid are discussed below.

Liver

Liver homogenates from females at the follicular stage demonstrated abundant amounts of several ketones: 6-methyl-5-hepten-2-one; 2,6,6-trimethyl-2-cyclohexanone; 4,4,6-trimethyl-2-cyclohexen-1-one; 3-buten-2-one 4-(2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1-yl (Table 3 and Figure 5). Significant amounts of the methyl ester of 1-methyl-2,5-cyclohexadien carboxylic acid and *n*-hexadecanoate (palmitate) were present (Figure 5).

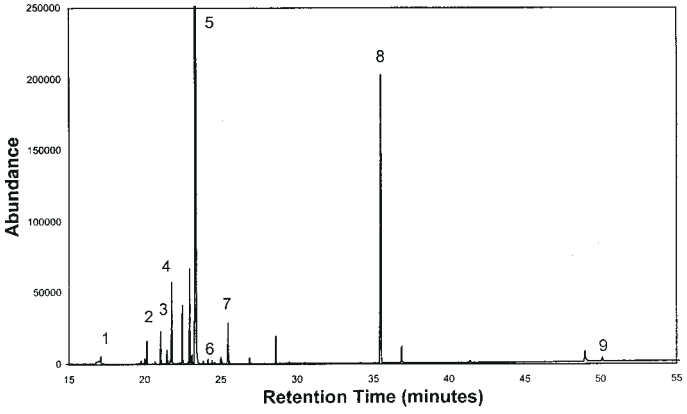

Figure 5 SPME–GC separation of liver homogenate. Peaks 1–9 are as follows: (1) cyclohexanol; (2) 6-methyl-5-hepten-2-one; (3) 6-methyl-5-hepten-2-ol; (4) methyl ester of 1-methyl-2,5-cyclohexadien carboxylic acid; (5) 2,6,6-trimethyl-2-cyclohexenone; (6) 4,4,6-trimethyl-2-cyclohexen-1-one; (7) dodecanal; (8) 3-buten-2-one 4-(2,2,6-trimethyl-7-oxabicyclo-[4.1.0]hept-1-yl); (9) methyl ester of tetradecanoic acid.

Discussion

Advantages of the ECC/CT–GC/MS and SPME–GC/MS techniques

The two headspace techniques used in conjunction with GC/MS to characterize and quantitate *Z*7–12:Ac in female Asian elephant urine and serum were complementary.

ECC/CT revealed many volatile compounds (C3–C14) in trace amounts in the urine and blood (Rasmussen, 1998; this study). SPME resolved compounds from acetaldehyde through C18 and was especially selective for certain types of compounds of higher molecular weight. Both techniques were quantitative and had the advantage of direct measurement with no solvent involvement. The possibility of chemical change during analysis was reduced and sensitivity losses during mass spectrometry were avoided. ECC/CT–GC/MS quantitation has been well substantiated (Rasmussen, 1998). Quantitation with SPME was possible with stringent precautions. The major advantage of SPME was its microtechnique. Whereas ECC/CT required 50 ml of urine, SPME was effective at both 1 ml and 10 µl volumes.

Long-term and short-term stability of *Z*7–12:Ac

Under field conditions the maximum signal lifetime for urinary *Z*7–12:Ac is 2–3 days; often within 24 h a diminution of signal intensity is evidenced by a reduction in male bioresponses. Factors that produce short-term and long-term changes either directly to *Z*7–12:Ac or to its carriers affect its lifetime as a chemical signal, i.e. its bioactivity. These effects may occur in the urine, truncal mucus or vomeronasal organ mucus, but this discussion focuses on changes that may occur to urinary *Z*7–12:Ac. The demonstrated stability of pure *Z*7–12:Ac in water media at various temperatures and for varying lengths of time indicated that the stability of *Z*7–12:Ac in a hydrolytic environment would be reasonably good. The aqueous medium of urine is only one factor; urinary *Z*7–12:Ac is affected by adsorption to substrates, binding to carriers, pH, temperature and enzymatic agents. Depletion of *Z*7–12:Ac, with corresponding increased (*Z*)-dodecenol, occurred during exhaustive extraction (using heat, vigorous mixing, pH changes and protease addition) or during a 3 day stimulated degradation period (three 12 h sessions). Heat affects the vapor pressure (Pa) of *Z*7–12:Ac, which is 0.33 Pa at 25°C, but increased volatilization occurs with higher temperatures, such as 37°C. In contrast, the vapor pressure of (*Z*)-dodecenol is only half that of the acetate (0.17 Pa at 25°C) (J. Lazar, personal communication). Alkaline pH favors a gradual hydrolysis from *Z*7–12:Ac to the corresponding alcohol. However, the more alkaline pH also favors increased binding of *Z*7–12:Ac to its urinary carrier, elephant albumin. Such increased binding may reduce the degree of hydrolysis. This phenomenon is evidenced by comparative quantitative analyses of the acetate and its alcohol and by the bioresponse frequencies exhibited by male elephants. The data in Table 6 support previous bioresponse data estimating a natural signal lifetime of 24–36 h (Rasmussen and Schulte, 1999). Data from stored pre-ovulatory urine samples frozen at –20°C demonstrated that the occurrence of ester hydrolysis to alcohol could be measured both chemically and by a diminution in bioactivity (Rasmussen and Schulte, 1999; Rasmussen and Goodwin, 2000).

Significance of the correlation of elevated pH, protein and *Z*7–12:Ac during the pre-ovulatory stage

Protein carriers have recently been described as playing significant roles in transport of the pre-ovulatory pheromone in the male Asian elephant prior to signal transduction in the vomeronasal organ (Lazar *et al.*, 2000). During the pre-ovulatory period urinary protein levels were elevated, making increased amounts of the albumin carrier available; a more alkaline environment created tighter ligand–albumin binding. These two factors facilitated an increased urinary holding capacity for the elevated *Z*7–12:Ac.

The presence of related compounds

In this study and others (Rasmussen *et al.*, 1996, 1997; Rasmussen, 1998) none of the related compounds were bioactive as single compounds nor did their combination with *Z*7–12:Ac enhance or reduce bioactivity. Rather, the amount of *Z*7–12:Ac and its binding to proteins was the important factor.

Urinary *Z*7–12:Ac concentrations during the estrous cycle

Table 5 and Figure 3 show the interrelationship between two reproductive hormones, progesterone and LH, and the concentration of pheromone during the estrous cycle. Even with daily serum sampling there was no apparent correlation between LH levels and pheromone concentration; rather, an inverse correlation was apparent between low levels of progesterone and elevated levels of pheromone. The two distinct elevations in serum LH levels, separated by a 3 week time span, were not correlated with any abrupt elevations in pheromone concentrations. Rather, pheromone concentrations elevated linearly although unevenly in the interval between the two LH peaks, and pheromone levels tended to decrease at the time of the second LH peak when progesterone levels were also rising as the luteal stage began (Figure 3). During the luteal phase *Z*7–12:Ac was not detectable. At the end of the luteal stage, when progesterone levels declined as the follicular stage began, low concentrations of *Z*7–12:Ac were measurable and a linear increase occurred throughout the follicular period.

The non-cycling female who attracted male elephants released copious amounts of pheromone continuously. This female possessed leiomyomas (smooth muscle tumors) throughout her urogenital tract (D. Schmitt, personal communication). While it is doubtful that these had any relationship to pheromone production, they might be an indication of either lack of estrogen or progesterone. Uterine leiomyomas in another elephant regressed completely during pregnancy (J. Brown, personal communication). The lack of progesterone cyclicity in the high pheromone producer does suggest a relationship between this progestin and pheromone levels.

Significance of the linear increase in the concentration of *Z*7–12:Ac

The linear increase in the concentration of *Z*7–12:Ac that occurs throughout the follicular stage is in distinct contrast to the abrupt transitory peaks exhibited by reproductively related serum hormones (such as LH) or by urinary steroid hormonal metabolites. The linear increase in *Z*7–12:Ac may be a chemical signaling strategy designed to maximize the chance of a male finding a female at the proper time to mate. A transitory peak, elevating in concentration for only short periods (<6 h), would not be an effective signal to facilitate Asian elephant mating. Even with a lifetime of 24 h the message in the signal might be sufficiently degraded by the time a male elephant located it to be useless in providing either estrous state or individual identity information.

The ecology of the Asian elephant, now and during evolution, has had a strong reciprocal effect on the development of pheromonal strategies (Rasmussen, 1999). An attractive alerting signal needs to be emitted constantly for a time period sufficient for the male elephant to localize females for two reasons: the sexes live somewhat physically separate and the female Asian elephant ovulates once during a 3 month long estrous cycle. An increasing concentration of pheromone would allow precise assessment of female reproductive state. This female signal has a dual purpose: to attract the male towards the vicinity of the female and to provide him with information on how close ovulation is. A message that gradually increases in concentration would give valuable information to an experienced male, allowing him to assess impending ovulation by flehmen responses. Multiple flehmen responses and comparative flehmen responses between the urine of several females would allow males to obtain precise and quantitative information. Among the male mammals that exhibit multiple flehmens giraffes (*Giraffa camelopardalis*) flehmened more when they were sexually active. They exhibited more repetitive multiple flehmens toward females on the days females were in heat (Dagg and Foster, 1982), again suggesting the requirement for precise information. For the male elephant constant pheromonal input may result not only in behavioral responses but also in primer pheromone effects.

Origin and possible synthetic pathways

As a first step towards the delineation of pheromone locale and its synthetic pathway several candidate tissues were searched. Surprisingly, urine-free urogenital mucus samples did not contain *Z*7–12:Ac. Although the mucus as a source of pheromone cannot be totally ruled out, a synthetic pathway via dodecanoic acid might be possible because of the presence of significant amounts of several carboxylic acids; however, such synthesis has not been described in mammals. In addition, maximal flehmen responses and other premating behaviors by males are significantly correlated with increased urinary pheromone concentrations and occur just prior to ovulation. In contrast, females exhibit maximal tail flicking (of urine and/or mucus droplets) during the mid follicular stage (Slade, 1999). In the wild males do not select females that are tail flicking predominantly mucus; instead, tail flicking is correlated with maximal urogenital checks by other females (Rasmussen, unpublished data). This observation is consistent with the lack of *Z*7–12:Ac in the mucus.

This lack of detectable *Z*7–12:Ac in the mucus and the large amounts of *Z*7–12:Ac in the urine suggest that blood might be a more feasible source of the urinary compound. Blood volatiles from pre-ovulatory female elephants, as analyzed by SPME followed by GC/MS, demonstrated significant amounts of *Z*7–12:Ac. These amounts were elevated when non-specific protease was added to the sample, suggesting release of *Z*7–12:Ac from its known albumin carrier. The amounts were sufficiently high in serum to account for the high time-dependent urinary levels.

The next question is what is the source of *Z*7–12:Ac in the circulatory system? Volatile compounds from liver homogenates of pre-ovulatory females consistently demonstrated significant amounts of *n*-hexadecanoate (the methyl ester of hexadecanoic acid). This compound is a potential precursor of *Z*7–12:Ac and two synthetic pathways can be postulated. In the first suggested pathway fatty acid intermediates could be reduced to alcohols and subsequently acetylated to acetates. Membrane lipids could be a potential source of such fatty acyl groups; such groups are also common components in insect pheromone glands where they occur as components of several glycerolipids, e.g. triacylglycerols, ethanolamine phosphatides and choline phosphatides (Bjostad *et al.*, 1987; Bjostad, 1989). In insect sex pheromone glands free fatty acids do not occur (Bjostad *et al.*, 1987). For the elephant it is not known whether *Z*7–12:Ac is synthesized from hexadecanoic acid or its ester, i.e. hexadecanoate (palmitate) or whether *Z*11-16:Acyl or *Z*7–12:Acyl is formed. The second postulated synthesis could be via the pathway present in sex pheromone glands of the cabbage looper moth, *Trichoplusia ni*. Experiments in insects using radiolabels demonstrated that *Z*7–12:Ac is not derived directly from hexadecanoate. Fatty acids are first acetylated to 11-16:Acyl; subsequently desaturation occurs, followed by two successive 2-carbon chain shortenings. In this insect species the *Z*11 desaturase is located microsomally and accepts hexadecanoyl or octadecanoyl CoA (but not tetradecanoyl CoA) with a requirement for NADH (but not NADPH) (Bjostad and Roelofs, 1983; Wolf and Roelofs, 1986). In insects the pH optimum for these reactions is 7.4–7.8. The pH optimums of both the *Z*-7 and *Z*-9 desaturatases are significantly lower than the alkaline pH preferred by the elephant ligand when it binds to albumin. However, the pH of the blood and even the urine in early follicular stages is ~7.4 (Rasmussen *et al.*, 1982) and this latter pathway of synthesis, especially in liver/blood, is conceivable.

In the female elephant it is possible that hormonal changes during the follicular stage may influence pheromone concentrations. In comparison, in rats small increments of estradiol can result in hypertriglyceridemia. This state can result from either or both stimulation of synthesis of triacylglycerol transporting proteins and/or increased hepatic triacylglycerol synthesis and secretion (Wilcox *et al.*, 1981). Small periodic variations in serum estradiol, especially during the follicular period, have been described in female elephants (Hess *et al.*, 1983; Hodges, 1998). These elevations could induce such hepatic triacylglycerol synthesis and thus result in increased *Z*7–12:Ac by the first pathway. As *Z*7–12:Ac is gradually elevated before the second serum LH peak, the influence of estradiol may be a more plausible explanation. As preliminary results indicate that both *n*-hexadecanoate (palmitate) and *Z*7–12:Ac may be present in ovarian tissue, our current studies are focused on individual ripening pre-ovulatory ovarian follicles.

Acknowledgements

Drs Janine Brown, Thomas E. Goodwin and David Hess conducted the reproductive hormone assays. Ringling Center for Elephant Conservation (Gary Jacobson and Jim Williams), the Riddle Elephant Sanctuary and the Oregon Zoo made elephants available for bioassays and provided urine and blood samples. Special thanks are due at the latter facility to Charles Rutkowski and Fred Marion. David Blasko, Marine World/Six Flags, provided samples of elephant ovary and liver. This research was supported in part by grant DC03320 from the National Institutes of Health and by Biophysics Research Corp.

References

Arthur, C.L. and **Pawliszyn, J.** (1990) *Solid phase microextraction with thermal desorption using fused silica optical fibers*. Anal. Chem., 62, 2145–2148.

Benedict, F.G. (1936) The Physiology of the Elephant. Carnegie Institution, Washington, DC, pp. 1–301.

Bjostad, L.B. (1989) *Chemical characterization of sex pheromones and their biosynthetic intermediates.* Chem. Senses, 14, 411–420.

Bjostad, L.B. and **Roelofs, W.L.** (1983) *Sex pheromone biosynthesis in* Trichoplusia ni*: key steps involve Δ-11 desaturation and chain shortening*. Science, 220, 1387–1389.

Bjostad, L.B., Wolf, W.A. and **Roelofs, W.L.** (1987) *Pheromone biosynthesis in Lepidopterans: desaturation and chain shortening*. In Prestwich, G.D. and Blomquist, G.J. (eds), Pheromone Biochemistry. Academic Press, Orlando, FL, pp. 77–117.

Brown, J.L. (2000) *Reproductive endocrine monitoring of elephants: an essential tool for assisting captive management.* Zoo Biol., 19, 347–368.

Brown, J.L., Citino, S.C., Bush, M., Lehnhardt, J. and **Phillips, L.G.** (1991) *Cyclic patterns of luteinizing hormone, follicle-stimulation hormone, inhibin, and progesterone secretion in the Asian elephant (*Elephas maximus*).* J. Zoo Wildl. Med., 22, 49–57.

Brown, J.L., Schmitt, D.L., Bellem, A., Graham, L.H. and **Lehnhardt, J.** (1999) *Hormone secretion in the Asian elephant (*Elephas maximus*): characterization of ovulatory and anovulatory luteinizing hormone surges.* Biol. Reprod., 61, 1294–1299.

Dagg, A.I. and **Foster, J.B.** (1982) The Giraffe. Its Biology, Behavior, and Ecology. Robert E. Krieger Publishing Co., Malabar, FL.

Hess, D.L., Schmidt, A.M. and **Schmidt, M.J.** (1983) *Reproductive cycle of the Asian elephant (*Elephas maximus*) in captivity.* Biol. Reprod., 28, 767–773.

Heistermann, M., Trohorsch, B. and **Hodges, J.K.** (1997) *Patterns of excretion of faecal progesterone and 5α-reduced progestins during the ovarian cycle and early pregnancy in the African elephant (*Loxodonta africana*).* J. Reprod. Fertil., 19, 57.

Hermes, R., Olson, D., Göritz, F., Brown, J.L., Schmitt, D.L., Hagan, D., Peterson, J.S., Fritsch, G. and **Hildebrandt, T.B.** (2000) *Ultrasonography of the estrous cycle in female African elephants (*Loxodonta africana*).* Zoo Biol., 19, 369–382.

Hodges, J.K. (1998) *Endocrinology of the ovarian cycle and pregnancy in the Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephant.* Anim. Reprod. Sci., 53, 3–18.

Hodges, J.K., Heistermann, M., Beard, A. and **van Aarde, R.J.** (1997) *Concentrations of progesterone and the 5-α-reduced progestins, 5-α-pregnane-3,20-dione and 3-α-hydroxy-5-α-pregnan-20-one, in luteal tissue and circulating blood and their relationship to luteal function in the African elephant,* Loxodonta africana. Biol. Reprod., 56, 640–646.

Kaputsin, N., Critser, J.K., Olson, D. and **Malven, P.V.** (1996) *Nonluteal estrous cycles of 3-week duration are initiated by anovulatory luteinizing hormone peaks in African elephants.* Biol. Reprod., 55, 1147–1154.

Lazar, J., Prestwich, G.D. and **Rasmussen, L.E.L.** (2000) *Urinary and trunk mucous protein carriers of (Z)-dodecenyl acetate, the sex pheromone of the Asian elephant.* Chem. Senses, 25, 24.

Perrin, T.E., Rasmussen, L.E.L., Gunawardena, R. and **Rasmussen, R.A.** (1996) *A method for collection, long-term storage, and bioassay of labile volatile chemosignals.* J. Chem. Ecol., 21, 207–221.

Poon, K.F., Lam, P.K. and **Lam, M.H.** (1999) *Determination of polychlorinated biphenyls in human blood serum by SPME.* Chemosphere, 39, 905–912.

Prestwich, G.D. (1987) *Chemical studies of pheromone reception and catabolism.* In Prestwich, G.D. and Blomquist, G.J. (eds), Pheromone Biochemistry. Academic Press, Orlando, FL, pp. 473–521.

Rasmussen, L.E.L. (1998) *Chemical communication: an integral part of functional Asian elephant (*Elephas maximus*) society.* Ecoscience, 5, 410–426.

Rasmussen, L.E.L. (1999) *Evolution of chemical signals in the Asian elephant,* Elephas maximus*: behavioral and ecological influences.* J. Biosci., 24, 241–251.

Rasmussen, L.E.L. and **Goodwin, T.E.** (2000) *Initial studies on the source and cyclic release pattern of (Z)-7-dodecenyl-acetate.* Chem. Senses, 24, 25.

Rasmussen, L.E.L. and **Krishnamurthy, V.** (2000) *How chemical signals integrate Asian elephant society: the known and the unknown.* Zoo Biol., 19, 405–423.

Rasmussen, L.E.L. and **Perrin, T.E.** (1999) *Physiological correlates of musth: lipid metabolites and chemosignal composition of exudates.* Physiol. Behav., 67, 539–549.

Rasmussen, L.E.L. and **Schulte, B.A.** (1999) *Ecological and biochemical constraints on pheromonal signaling systems in Asian elephants and their evolutionary implications.* In Johnston, R.E., Muller-Schwarze, D. and Sorenson, P.W. (eds), Advances in Chemical Communication in Vertebrates 8. Kluwer Academic/Plenum Press, New York, pp. 49–62.

Rasmussen, L.E.L., Schmidt, M.J., Henneous, R., Groves, D. and **Daves, G.D. Jr** (1982) *Asian bull elephants: flehmen-like responses to extractable components in female elephant estrous urine.* Science, 217, 159–162.

Rasmussen, L.E.L., Lee, T.D., Roelofs, W.L., Zhang, A. and **Daves, G.D. Jr** (1996) *Insect pheromone in elephants.* Nature, 379, 684.

Rasmussen, L.E.L., Lee, T.D., Zhang, A., Roelofs, W.L. and **Daves, G.D. Jr** (1997) *Purification, identification, concentration and bioactivity of (Z)-7-dodecen-1-yl acetate: sex pheromone of the female Asian elephant,* Elephas maximus. Chem. Senses, 22, 417–437.

Rasmussen, L.E.L., Lazar, J., Greenwood, D., Feng, L. and **Prestwich, G.D.** (1998) *Initial characterizations of secreted proteins from Asian elephants that bind the sex pheromone, (Z)-7-dodecenyl acetate.* Chem. Senses, 23, 591.

Rasmussen, L.E.L., Lazar, J. and **Prestwich, G.D.** (1999) *Initial molecular studies of (Z)-7-dodecenyl acetate as a mammalian sex pheromone.* Chem. Senses, 24, abstract 365.

Schulte, B.A. and **Rasmussen, L.E.L.** (1999) *Signal–receiver interplay in the communication of male condition by Asian elephants.* Anim. Behav., 57, 1265–1274.

Schwarzenberg, F., Straus, G., Hoppen, H.-O., Schaftenaar, W., Dieleman, S.J., Zenker, W. and **Pagan, O.** (1997) *Evaluation of progesterone and 20-oxo-progestagens in the plasma of Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants.* Zoo Biol., 16, 403–413.

Slade, B.E. (1999) *Chemical communication of estrous cycle status and change in social behaviors with reproductive state in captive female Asian elephants,* Elephas maximus, Masters Thesis, Portland State University, Portland, OR.

Wilcox, H.G., Kenagy, R., Weinstein, I. and **Heimberg, M.** (1981) *Alterations of plasma HDL lipids and apolipoproteins in female rats treated with ethynyl estradiol.* Biochim. Biophys. Acta, 666, 348–355.

Wolf, W.A. and **Roelofs, W.L.** (1986) *Properties of the Δ-11 desaturase enzyme used in cabbage looper moth sex pheromone biosynthesis.* Arch. Insect Biochem. Physiol., 3, 45–52.

Yamazaki, K., Beauchamp, G.K., Singer, A., Bard, J. and **Boyse, E.A.** (1999) *Odor types: their origin and composition.* Proc. Natl Acad. Sci. USA, 96, 1522–1525.

Accepted February 27, 2001