Purification, Identification, Concentration and Bioactivity of (*Z*)-7-Dodecen-1-yl Acetate: Sex Pheromone of the Female Asian Elephant, *Elephas maximus*

L.E.L. Rasmussen, Terry D. Lee[1], Aijun Zhang[2], Wendell L. Roelofs[2] and G. Doyle Daves Jr[3]

Department of Chemistry, Biochemistry, and Molecular Biology, Oregon Graduate Institute of Science and Technology, Portland, OR 97291-1000, [1]Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, CA 91010, [2]Department of Entomology, Cornell University, Geneva, NY 14456 and [3]Department of Chemistry, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

Correspondence to be sent to: L.E L Rasmussen, Oregon Graduate Institute, PO Box 91000, Portland, OR 97291-1000, USA

Abstract

In their natural ecosystems, adult male and female Asian elephants, *Elephas maximus*, live separately. For several weeks prior to ovulation, female elephants release a substance in their urine which elicits a high frequency of non-habituating chemosensory responses, especially flehmen responses, from male elephants. These responses occur prior to, and are an integral part of, mating Using bioassay-guided fractionation, quantitatively dependent on these chemosensory responses, a specific sex pheromone was isolated and purified by an alternating series of organic and/or aqueous extractions, column chromatography, gas chromatography and high-performance liquid chromatography Using primarily ^{1}H-proton nuclear magnetic resonance (NMR) spectrometry and gas chromatography–mass spectrometry (GC–MS) of the urine-derived pheromone and its dimethyl disulfide derivative, we determined the structure of the active compound to be (*Z*)-7-dodecen-1-yl acetate (*Z*7-12:Ac). Concentrations of *Z*7-12.Ac in the female urine increased from non-detectable during the luteal phase to 0.48 μg/ml (0.002 mM) early in the follicular phase and to 33.0 μg/ml (0.146 mM) just prior to ovulation. Bioassays with commercially available authentic synthetic *Z*7-12:Ac, using 10 Asian male elephants at several locations in the US, demonstrated quantitatively elevated chemosensory responses that were robust during successive tests, and several mating-associated behaviors. Bioassays with *Z*7-12:Ac with adult male elephants dwelling in more natural social situations in forest camps in Myanmar revealed some differing contextual pre-mating behavioral components. The remarkable convergent evolution of this compound suggests that compounds identified in mammalian exudates that are also present in pheromone blends of insects should be re-evaluated as potential mammalian chemosignals.
Chem. Senses 22: 417–437, 1997.

Introduction

In mammals, the study of perceptual mechanisms of pheromonal cueing in relationship to complex sexual behaviors has been hampered by the lack of identification of specific compounds functioning as conspecific chemical signals. Only for a few species of mammals, such as mice and hamsters, has an active dialog been possible among behavioral, chemical and neurophysiological aspects of pheromonal communication relative to sexual reproduction (Wysocki, 1979; Singer *et al.*, 1984a,b, 1986, 1988; Singer, 1991; Singer and Macrides, 1993). Multidisciplinary studies as exemplified by investigations of insects (Roelofs and Brown, 1982; Roelofs, 1995) and fish (Sorensen, 1992a,b) have not been achieved for mammals with more complex behavioral repertoires. There are incredible challenges to studying chemical signals in animals that live in sophisticated societies and are capable of a high degree of learned behavior; yet these chemical signals can send powerful behavioral messages of considerable social importance. For example, some animals have the ability to modulate behavioral responsiveness to particular pheromones as a result of learning processes (Wysocki *et al.*, 1986). The animal must specifically, and presumably instinctively, recognize the signal and follow it in a complex, three-dimensional, and chemically noisy environment. Therefore, chemical identification of a released chemical signal that elicits specific responses and/or behaviors will allow tracking of this emission and elucidation of its receptor mechanisms. The presence of a female-to-male sexual pheromone in Asian elephants, *Elephas maximus*, was clearly indicated by chemosensory responses and pre-mating behaviors of adult males to female urinary cues. The appearance of this chemical signal occurred during a specific period in the female reproductive cycle, and the male response presented an unusual opportunity to observe and quantitate this signal by bioassays.

In the design of this study, it was necessary to consider the context of how pheromones function at the behavioral level, as this is embedded in the social fabric of a particular species (Rasmussen, 1997). Among Asian elephants, the familial social organization is matriarchal, consisting of relatively small family herds of female offspring (Eisenberg *et al.*, 1971; Buss, 1990). Adult male Asian elephants are largely solitary or in small transitory male groups (Eisenberg *et al*, 1971; Sukumar, 1989). These males are often in proximity to the female groups, but usually not intimately associated with them. Most female Asian elephants cycle throughout the year, exhibiting a uniquely long estrous cycle. This cycle varies from 12 to 18 weeks in length, averaging 16.3 ± 0.4 [mean ± SEM (standard error of mean)]. The follicular phase has a mean of 6 weeks (Hess *et al.*, 1983). Males in the natural forest habitat apparently locate the females and detect the proper time for mating through the chemical senses (Eisenberg *et al.*, 1971).

We have established that during estrus, and especially prior to ovulation, female Asian elephants release a urinary pheromone that elicits high frequencies of several types of chemosensory responses from male elephants (Rasmussen *et al.*, 1982, 1986, 1993). Three chemosensory responses—checks, places and flehmens—are overt, readily quantifiable, and non-habituating. The flehmen response is the most definitive and is an integral part of the mating scenario (Rasmussen *et al.*, 1986). Check and place responses occur prior to flehmens and as an integral part of flehmens or as independent responses. The flehmen response is proceeded by sniffs and checks and/or places. The flehmen is the penultimate chemosensory response prior to mating behavior. During the flehmen response, liquids containing compounds eliciting the response (elicitors) are transported by the trunk tip finger (after checks and/or place responses) to the paired openings of the ducts of the vomeronasal organ in the anterior region of the hard palate (Rasmussen and Hultgren, 1990; Rasmussen *et al.*, 1993; Rasmussen and Munger, 1996). Thus, the flehmen response is a discrete motor pattern exhibited specifically during the sensory evaluation of chemicals. The definitive flehmen response formed the basis for a specific and quantitative bioassay (Rasmussen *et al.*, 1982, 1986, 1993). Subsequent to chemoresponses (primarily multiple flehmen responses evoked by pre-ovulatory urine), male sexual arousal behaviors may occur. These include erections and penile searching motions (Eisenberg *et al.*, 1971; Rasmussen *et al.*, 1986, 1993) and, if the female is available, mounting and copulation.

We utilized these chemosensory responses, primarily the flehmen responses, to direct the isolation of the bioactive agent, i.e. the pheromone. The active agent(s) was demonstrated to be organic-solvent extractable (Rasmussen *et al.*, 1982). Progressive purification of the active principle was guided by quantitative bioassays using several chemosensory responses (Rasmussen *et al.*, 1982, 1986, 1993). Now

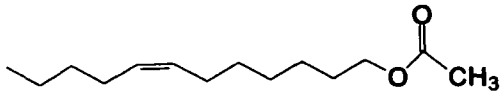


Figure 1 Structure of the elephant female-to-male sex pheromone, (*Z*)-7-dodecen-1-yl acetate (*Z*7-12 Ac).

we report in detail the modifications of our previous purification methodology that has resulted in the final isolation and purification. Furthermore, this principle has been unambiguously identified in the pre-ovulatory urine of the estrous female Asian elephant as (*Z*)-7-dodecen-1-yl acetate (*Z*7-12:Ac) (Figure 1) (Rasmussen *et al*, 1996). Most essentially, the bioactivity of the authentic synthetic (*Z*)-7-dodecen-1-yl acetate has been established by exhaustive, repetitive testing of Asian male elephants, both solitary males in captive facilities within the United States and more recently males of varying social positions residing in hardwood lumber camps of southeast Asia. This report, with its details of our purification and identification methodologies, its informative new data on the concentration patterns of the identified compound during the estrous cycle, and the fascinating account of not only the sustained responses of solitary captive males but the differential responses of semi-wild males in relationship to their dominance status, represents a significant contribution to our understanding of the role of chemical senses in the reproductive behavior of the Asian elephant.

Materials and methods

Separation and purification methods

Urine was collected from nine mature female Asian elephants during the appropriate pre-ovulatory days as determined by measurement of serum progesterone concentrations, by assessment of cervical mucus, and by monitoring daily the responses of males to females (Hess *et al.*, 1983). Serum progesterone was measured by standard radioimmunoassay procedures at laboratory facilities at the Metro Washington Park Zoo (Hess *et al.*, 1983). Control anestrous urine samples were obtained during the luteal phase of the cycle.

The procedure employed to isolate the pheromone from pre-ovulatory urine and purify it for chemical analyses utilized a series of fractionation steps previously described (Rasmussen *et al.*, 1993) with modifications that have improved the yields at least 10-fold (Figure 2).

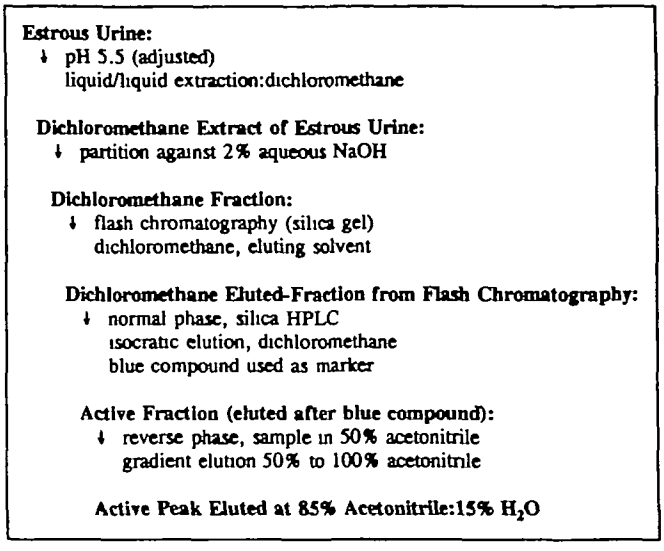


Figure 2 Extraction and purification scheme for the estrous pre-ovulatory pheromone of the female Asian elephant The bioactivity remained in the organic phase, although a large amount of highly colored inactive material was removed with 2% aqueous sodium hydroxide partitioning. Sequential normal and reverse-phase high-performance liquid chromatography (HPLC) used for the final purification removed minor contaminants and resulted in an active fraction eluting at 85% acetonitrile with a single dominant component

The active component was separated and purified from the pre-ovulatory urine by the procedure outlined in Figure 2. Pre-ovulatory urine (pH adjusted to 5.5) was extracted with dichloromethane using four 5 l capacity liquid–liquid extractors. The ratio of extracting solvent to urine was 1:4. The dichloromethane extract was concentrated 20-fold (100 to 5 l). The concentrated extract was then partitioned against 5 l of 2% sodium hydroxide at 20°C. The resultant highly colored aqueous sodium hydroxide phase was inactive at alkaline, neutral and acidic pH, and was discarded (see also Rasmussen *et al.*, 1986). The bioactive organic solution was then concentrated another 50-fold (5 l to 100 ml) and fractionated by flash chromatography, using EM silica gel 60 particle size (0.040–0.063 mm) and dichloromethane as eluent (Still *et al.*, 1978; Rasmussen *et al.*, 1982). The activity was localized in a blue-colored fraction. All other fractions were not bioactive (Rasmussen *et al.*, 1982, 1986, 1993). This blue substance, present in estrous urine, was subsequently employed as a marker to standardize fractions during flash chromatography and the next purification step of normal phase high-performance liquid chromatography (HPLC). The HPLC fractionation step was reliable, provided the blue substance was utilized as a marker to standardize fraction selection. The column used for normal phase HPLC was a Whatman microparticulate

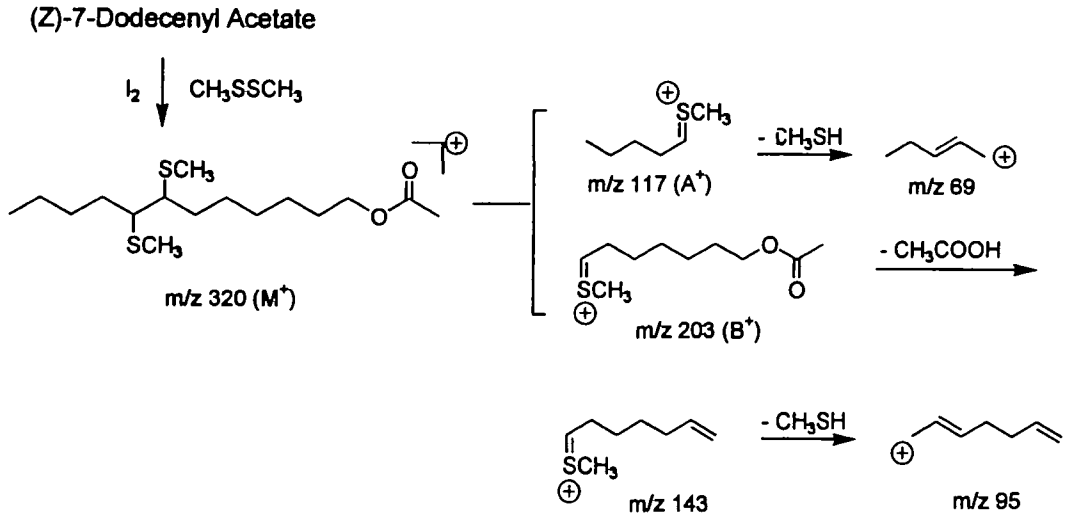


Figure 3 The technique of dimethyl disulfide (DMDS) adducts of *Z*7-12 Ac, demonstrating degradation pathways and derivatization of an olefin with DMDS for determining position of the double bond

silica gel, Partisil 10 (Magnum 9), 25 × 2.5 cm. Elution was isocratic; the elution solvent was dichloromethane. The flow rate, 4 ml/min, was maintained using Waters Model 501 dual piston pumps. Detection was accomplished using a Waters 440 ultraviolet detector set at three wavelengths: 225 or 280 and 340 nm. After the initial tentative identification of a dodecenyl, all subsequent absorption measurements were conducted at 280 nm. Indeed, the pheromone only exhibits very weak absorption at ~270 nm.

Final purification used a reverse-phase HPLC fractionation step on a Partisil 10 analytical column (25 × 1 cm) and linear gradient elution of 1.4 ml/min beginning with 50% acetonitrile:50% water to a final eluent concentration of 100% acetonitrile. The active band eluted at 85% acetonitrile:15% water. All other fractions assayed negative. The active fraction obtained from this procedure was sufficiently pure to allow mass and nuclear magnetic spectrometric (NMR) analyses.

Analytical and structural characterization

The progressive purity of the latter stage active fractions was monitored by gas chromatography (GC). When the pure active substance was obtained, the structure was elucidated using ^{1}H-proton NMR and mass spectrometries and appropriate chemical procedures.

Purified samples of consistent bioactivity were subjected to mass spectrometric analyses. Electron ionization (EI) mass spectra of the purified pheromone were obtained using a JEOL HX100HF mass spectrometer operating at 5 kV acceleration potential and a nominal resolving power of 500 for FD and 5000 for EI spectra. For EI spectra, the sample was placed on the outside of a short melting point capillary and inserted into the 150°C ion source using the direct insertion probe. A 70 eV electron beam was used for sample ionization.

Nuclear magnetic resonance

The 1H-NMR spectra were acquired on a Varian Unity+ 500 at a frequency of 500 MHz using a 5 mm indirect detection probe. The ^{13}C-NMR experiments were performed on a Varian Unity 300 spectrometer at a frequency of 75 MHz using a 5 mm broadband switchable probe (^{15}N to ^{31}P). The various samples were dissolved in 0.7 ml of CD_2Cl_2 (99% purity; Aldrich, Milwaukee, WI) and all experiments were run with the probe air temperature set at 25°C. Spectral widths of 6000 and 16 000 Hz were used for the ^{1}H and ^{13}C spectra respectively. The ^{13}C spectra were collected with WALTZ broadband proton decoupling, a second relaxation delay, and a 0.3 s acquisition time. Typically, 3000–5000 transients were co-added. The proton spectra, which were integrated, were run with a 10 s relaxation delay with 64–128 transients co-added. The processed spectra were zero-filled to 64 K points with 1–3 Hz of line broadening applied.

Dimethyl disulfide derivatization and GC–MS analysis of purified active elephant material

The dimethyl disulfide (DMDS) derivative was prepared as described by Buser and co-workers (1983). The pheromone preparation was dissolved in 0.4 ml of hexane. This solution

(50 µl) was then treated with 100 µl of DMDS (98% purity; Aldrich) and one drop of iodine solution (60 mg iodine in 1 ml diethyl ether). The reaction mixture was held in a 40°C oven for 24 h, cooled, and diluted with 200 µl hexane. Figure 3 depicts the reactions in the technique. Iodine was removed by shaking with 3 drops of 5% aqueous $Na_2S_2O_3$. The organic phase was removed, and the aqueous phase was extracted with 100 µl hexane. The combined hexane solution was dried over Na_2SO_4, concentrated to a small volume under nitrogen (~100 µl), and kept frozen for future analysis.

GC–MS was carried out with a Hewlett–Packard 5890 gas chromatograph coupled to an HP 5970B Mass Selective Detector using a polar SLP SILAR-10 C capillary column (50 m × 0.25 mm ID, 0.25 µm film thickness; QUADREX Corporation, New Haven, CT). The oven temperature was held at 100°C for 2 min, then programmed at 10°C/min to 220°C and held there for 30 min. The carrier gas was helium at a flow rate of 2 ml/min. Mass spectral analyses of the derivatives were used to confirm the molecular weight, locate the double bond, and determine the presence of either *Z* or *E* isomers.

Quantitative analyses of *Z*7-12:Ac in urine of cycling female elephants

Quantitative analysis of *Z*7-12:Ac in urine of cycling female elephants was performed by the solid phase microextraction (SPME) technique. A 5 ml aliquot of urine obtained from different stages was placed into a 7 ml glass vial. Standard heptadecane in hexane (1 µg/µl), 5 µl, was then added into each urine sample along with a micromagnetic stir bar. The solution was stirred at room temperature for 2 min with a fused silica fiber (100 µm non-bonded polydimethylsiloxane phase fiber installed in the SPME manual fiber holder; Supelco Inc., Bellefonte, PA) immersed in the solution. The SPME syringe then was injected into the GC injection port and the plunger pushed down to desorb the fiber for 5 min while running the GC program. An analysis of the detector response to hexadecane and *Z*7-12:Ac standards (1 ng/µl each) showed that the response areas were close to 1. All analyses were performed on a Hewlett–Packard 5880 gas chromatograph equipped with a polar Stabilwax capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness; Restek Corporation, Bellefonte, PA). The oven temperature was programmed at 100°C for 2 min, then 15°C/min to 220°C and held there for 30 min. The carrier gas was helium at a flow rate of 2 ml/min. The urinary concentration of *Z*7-12:Ac was measured at four hormonally distinctive periods during the female estrous cycle: luteal, early follicular, mid-follicular, and immediately prior to ovulation. Male response to female urine was quantitatively distinct during these periods (Rasmussen *et al.*, 1993). These periods are characterized in the female Asian elephant by serum progesterone concentrations (Hess *et al.*, 1982). Luteal phase progesterone levels for the particular female whose urine was assessed were >300 ng/ml. In contrast, the early follicular stage was characterized by decreasing serum progesterone levels, ranging only between 250 and 100 ng/ml. Mid-follicular levels were constant, ranging only between 50 and 100 ng/ml. The pre-ovulatory period was characterized by the lowest serum hormone levels and by initial elevations in serum progesterone levels as the cycle was completed and ovulation occurred. Serum estradiol levels in Asian elephants are often just above the detection limit and thus are not utilized to characterize the estrous cycle.

Qualitative characterizations of *Z*7-12:Ac in headspace volatiles of pre-ovulatory urine

One liter of pre-ovulatory (freshly collected and bioassayed active) urine and 1 l of luteal phase (freshly collected and bioassayed inactive) urine were heated to elephant body temperature (35.9°C) in separate special closed stainless-steel (ss) domed chambers. Prior to initiation of the controlled warming, manufactured purified air was flushed through each 3 l system; the system was then sealed and heated at 36°C for 30 min. Headspace volatiles for both the experimental and control samples were collected in an evacuated 6 l ss receiving canister. Subsequently, GC–MS analyses were conducted to provide identification of compounds from C_3 through C_{14} at concentrations as low as 0.10 parts per billion volume (ppbv) using the method described in detail by Perrin *et al.* (1996).

Bioassay protocol

Guides to bioactivity during purification and identification procedures

The flehmen response by the Asian male elephant is a discrete, all-or-none response that can be quantified to meet the specificity and quantitative requirements of a reliable

bioassay (Rasmussen *et al.*, 1982). The male elephant utilizes this response to determine the estrous state of a female elephant, and we have used it as a guide to fractionation protocol during purification of the bioactive component. Because habituation to estrous urine or its active components does not occur, successive bioassays during the fractionation procedures were possible (Rasmussen *et al.*, 1986, 1993).

During the procedures to isolate an unknown, yet biologically active substance, a measure of bioactivity was required to provide a degree of quantitation to the purification methodology. Accordingly, one bioassay unit was defined and standardized as equivalent to 500 ml of bioactive pre-ovulatory urine eliciting seven flehmen responses/h (median 7.0 [2.8—8.3 (25–75 percentile)]; mean 7.0 ± 0.49 flehmen responses/h) (Rasmussen *et al.*, 1982, 1986, 1993). Using this standard unit during each bioassay step ensured a definitive bioassay result to guide the continued purification. This bioassay unit was significantly higher than either control or baseline levels or novel substance response levels. [The novel substance responses are responses of moderately high frequency in initial bioassays (3–6 responses/h) which generally decrease to zero by the third test (Rasmussen *et al.*, 1986). Flehmen responses most definitely exemplify novel substance responses.] Novel substance responses occur when an elephant is tested with compounds or mixtures that have not been previously encountered either singly or in a particular combination. Because of this phenomenon, it is necessary to conduct multiple repetitive testing (three to five bioassay trials) to distinguish this novel substance response from sustained positive responses elicited by the estrous pheromone.

The primary test site, Metro Washington Park Zoo, Portland, OR, the elephants and the bioassay procedure have been described in detail elsewhere (Rasmussen *et al.*, 1982, 1986). For a number of years during the establishment of purification protocols, the bioassays were conducted almost exclusively at this site. Four males, between 11 and 34 years of age, were available for bioassay testing on a random rotation basis. Three were experienced breeders; response frequencies were somewhat, but not significantly, higher for the more naive male. Test samples (both the chromatographic fractions and *Z*7-12:Ac after identification) were dissolved in 3 ml acetone which was then added to 500 ml of appropriate buffered aqueous solutions or water. The final acetone concentration was 0.1 M, both in these assays and with subsequent assays of pure compounds. Fractions were assayed at physiological amounts, based on standardized bioactivity units (Rasmussen *et al*, 1986). (As described in the next section, *Z*7-12:Ac was assayed from 0.5 to 5.0 mM concentration in the same media.) Samples for each assay included appropriate controls including female anestrous urine and its extracts, solvents used for extraction and purification, and synthetic versions of compounds identified in estrous urine. The bioassays had extra controls. In the sandy regions of the exercise yard, naturally occurring urination spots from female elephants that were present. Chemosensory responses to these areas were also recorded. In addition, since male elephants were routinely let into the exercise yard without any bioassay samples being placed, baseline flehmen response frequencies were readily obtained.

Specifically, the bioassay was a focal-animal sampling technique (Altmann, 1974), involving a solitary free-roaming male elephant. Samples were placed at random in previously washed areas in the yard. The test animal detected these randomly placed samples during a 60-min observation period (Rasmussen *et al.*, 1982, 1986). Flehmen, check, and place responses, as well as other noteworthy actions, were scored. A minimum of two categories of controls, in addition to the natural urine spots, were included during each bioassay. These included appropriate concentrations of extractants and HPLC fractions adjacent to the active region. By bioassaying separated fractions, bioactivity was localized. The entire fractionation sequence was monitored by bioassays.

Control samples included solvents prepared at the same concentrations utilized during extraction procedures and preparations of anestrous female urine. These preparations were extracted, separated and purified by the same methodology employed for the purification of *Z*7-12:Ac from pre-ovulatory urine. Relevant fractions were examined for *Z*7-12:Ac using analytical HPLC and GC–MS. No *Z*7-12:Ac was detected. Aliquots of pre-HPLC preparations were 'spiked' with authentic *Z*7-12:Ac to ensure the precise localization of the acetate fraction during the subsequent HPLC fractions.

Occasionally during the isolation process, additional assays of fractions, partially purified material, and ultimately the compound purified from estrous urine were conducted under similar conditions with seven other male Asian elephants at other locations, including Dickerson Park Zoo, Springfield, MO, and Ringling Center for Elephant Conservation, Polk City, FL.

Assessment of bioactivity of authentic synthetic Z7-12:Ac

After Z7-12:Ac was identified as the principal component of the active elephant preparation, a systematic series of bioassays similar in design to the preceding assays was carried out to determine if the synthetic version of this compound, by itself or in selected combinations, consistently elicited non-habituating chemoresponses and was responsible for the observed behavioral responses. Five categories of experimental tests were conducted with the same procedures and media as outlined previously. Controls were assayed during each test. Unless specified otherwise, the tests (Experiments 1, 2, 4 and 5) were conducted with Z7-12:Ac [containing traces (~2.5%) of the *E* isomer] obtained commercially (Sigma Chemical Co., St Louis, MO). A series of assays (Experiment 3) was conducted with ultrapure (Z)-7-dodecen-1-yl acetate, provided by Dr Jose Tercio Ferreira, Universidade Federal de San Carlos, Brazil, and with *Z* and *E* isomers separately, purchased from Simon Voerman, The Netherlands.

Experiment 1: bioactivities of synthetic Z7-12:Ac compared with elephant-derived Z7-12:Ac at several concentrations

Authentic synthetic Z7-12:Ac and elephant estrous urine-derived Z7-12:Ac were compared in seven replicate tests involving nine solitary adult male Asian elephants of known maturity and varying breeding experience. A 10-fold concentration range (0.5–5.0 mM) synthetic Z7-12:Ac was tested with five males. All five males were tested between 0.5 mM and 1.0 mM. One male was also tested at 2.0 mM. Three males were also tested between 0.5 and 5.0 mM. Four additional males (nos 6–9) were tested with 1.0 mM Z7-12:Ac, as were three Asian female and three African (Loxodonta africana) male elephants. The latter animals were at two locations. They were sexually but not socially mature and were not proven breeders, but represent the only African males available for bioassay at the time. Control substances included identified aldehydes present prior to the final purification step, hexanal, heptanal, octanal, decanal and undecanal, and solvents used during fractionation or bioassay (dichloromethane, acetonitrile and acetone). Data were expressed as mean ± SE. The statistical test used was one-way ANOVA on ranks, repeated measures. In certain instances when two groups were compared, paired *t*-tests were used. In cases where zero responses were involved, data were re-calculated as medians (25–75th percentile); non-parametric statistics were used, including Kruskal–Wallis one-way ANOVA on ranks.

Experiment 2: multiple successive bioassays at constant concentration of Z7-12:Ac

Solitary males in captivity in the US continued to be assayed. Additional assays were conducted at 1.0 mM concentration. The results of trials 1–3 and 4–10 were compared and statistically significant differences assessed by one-way ANOVA on ranks. When required, medians and non-parametric statistics (Kruskal–Wallis one-way ANOVA on ranks) were used.

Experiment 3: comparison of bioactivity of (Z) and (E) isomers of 7-dodecenyl acetate

Three tests were conducted with five male elephants to assess the comparative bioactivity of the (Z) and (E) isomers of 7-dodecenyl acetate. These bioactivities were compared with bioactivity levels from whole pre-ovulatory urine, purified extracts from estrous urine, and non-ultrapure (Z)-7-dodecenyl acetate. Similar statistical tests were employed.

Experiment 4: efficacy assessment of different presentation media

Three different media—water, 0.01 M acetate buffer (pH 6.0), and anestrous urine—were assessed for any enhancement or inhibition of chemoresponse levels. For consistency and because of the relative water insolubility of Z7-12:Ac, the acetate was dissolved in acetone (final concentration of acetone, 0.1 M) prior to its addition to the bioassay media. Presentation in water allowed only effects elicited by Z7-12:Ac to be assessed. Buffer presentation may indicate possible pH effects; suspension in anestrous urine mimicked the natural situation.

Experiment 5: testing of multiple male groups in southeast Asia

Assays of synthetic Z7-12:Ac (final concentration 1.0 mM as dissolved in 3 ml of acetone and added to 500 ml distilled water) were conducted on three groups of mature working male elephants in the lumber camps of central Myanmar. Bioassays with the adult male Asian elephants in

Table 1 Organics and *Z*7-12 Ac/l of pre-ovulatory urine

Fraction	g/l in bioactive pre-ovulatory urine[a]	
	Total organics	*Z*7-12 Ac
Whole urine	50 g	33 µg (0.146 mM)
Solvent extract	50 mg	<5 µg (<0 002 mM)
Organic phase after NaOH partition	0.10 mg	nm[b]
Flash chromatography	1 mg	nm
Normal phase HPLC	100 µg	nm
Reverse phase HPLC	10 µg	nm[c]

[a]Concentrations are expressed as mass/volume because during the purification stages the identity and thus the mass weight of the active component was unknown. After *Z*7-12·Ac was identified, molar concentrations could be calculated and thus alternate units are listed

[b]nm = not measured

[c]100 l of starting urine was required to have sufficient bioactivity during final fraction steps for multiple bioassays Data indicate that progressive purification resulted in a 1000-fold reduction in organics at initial solvent extraction and between 10 and 25-fold reduction in each successive step Almost all of the *Z*7-12 Ac was lost during the initial extraction

Myanmar were conducted as multiple animal assays and under 'normal' conditions for the elephants at a particular village.

In this report, data are reported as mean ± SEM. Mean response values represent the number of times an elephant exhibited a particular chemosensory response or behavior in a 1 h time period. The mean is the value over the number of trials. When data were parametric and zeros were not involved, one-way ANOVA on ranks, repeated measures statistical analyses were employed. At times paired samples were compared by t-tests and were utilized in instances when data were parametric. Non-parametric data of high variance were re-expressed as median (25–75th percentile) and analysed by one-way ANOVA and Mann–Whitney rank sum or Kruskal–Wallis one-way ANOVA on ranks as appropriate. In non-response categories, i.e. where results were zero, non-parametric statistics using ranks, or the differences between experimental and control, were employed and tested to see if the median of the difference was significantly different from zero.

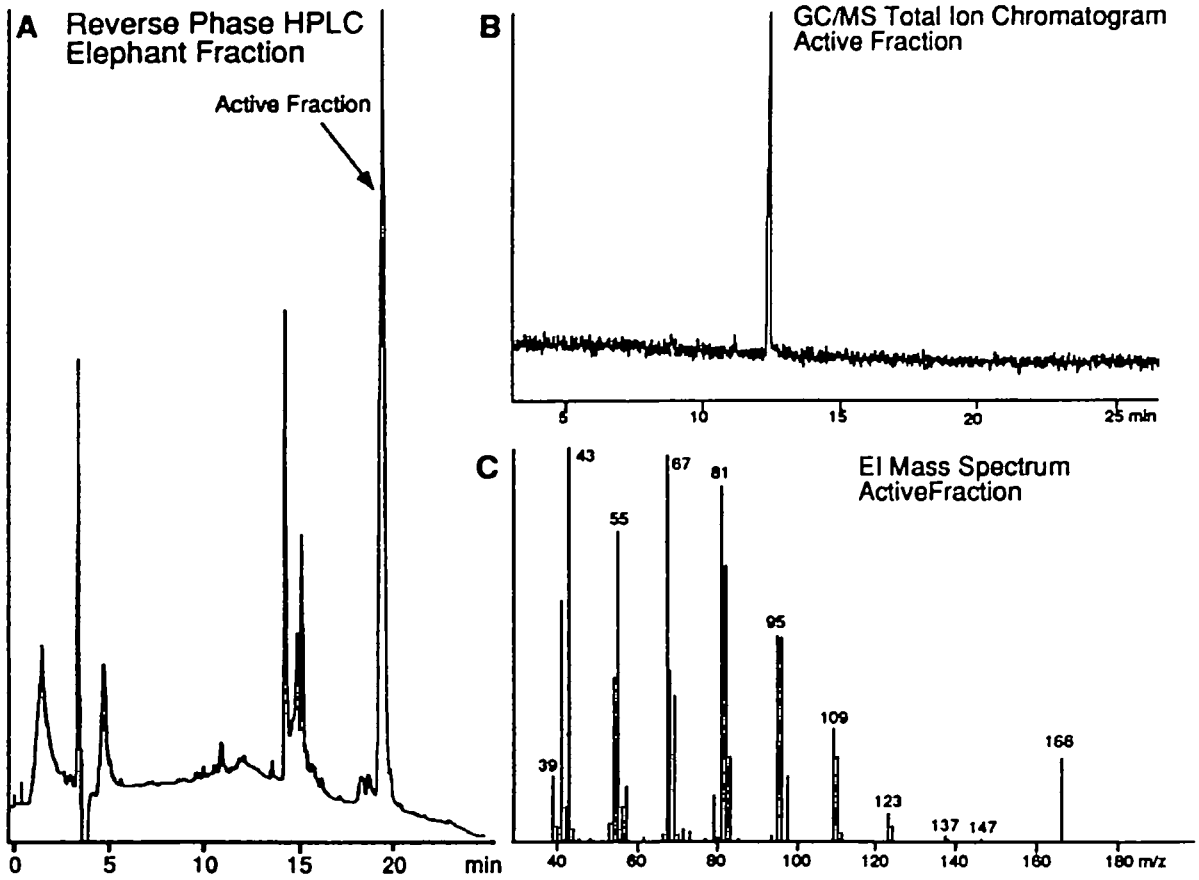


Figure 4 **(A)** Reverse-phase high-performance liquid chromatography (HPLC) using 50% acetonitrile/water as an initial solvent A linear gradient elution to 100% acetonitrile was employed in the final purification of the elephant pheromone which was contained in the dominant peak eluting at 20 min **(B)** Ion chromatogram of the active fraction **(C)** The electron ionization mass spectrum of the active fraction

Results

Identification

At each step during the progressive fractionation and purification, bioassay tests monitored the location and substantiated the continued presence of the bioactivity. Organic content in the active fraction was estimated based on weight (mass/volume) (Table 1). Recent additional extraction/fractionation steps, as compared with previous methodology (Rasmussen *et al.*, 1993) improved both the yield and purity of the active component. The addition of a final reverse phase HPLC step using gradient elution accomplished an increase in purity with minimal loss of bioactivity, although subsequently the yield was determined to be very low (Table 1).

A major late-eluting band from the final HPLC fractionation (Figure 4A) consistently tested positive in the bioassay. By GC–MS analysis, this fraction was a single component (Figure 4B) with an apparent molecular ion at *m/z* 166 (Figure 4C). The spectrum was consistent with a

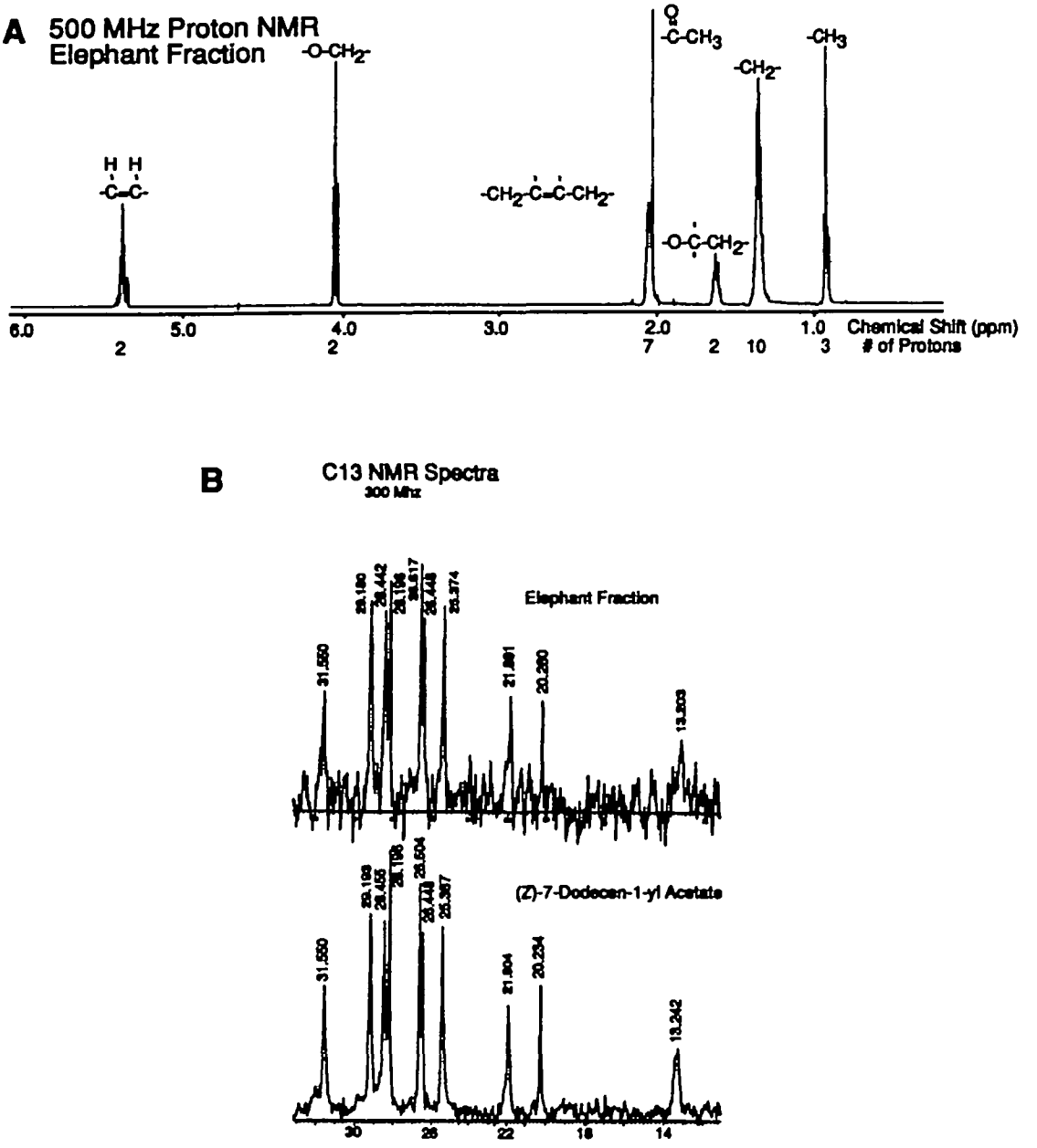
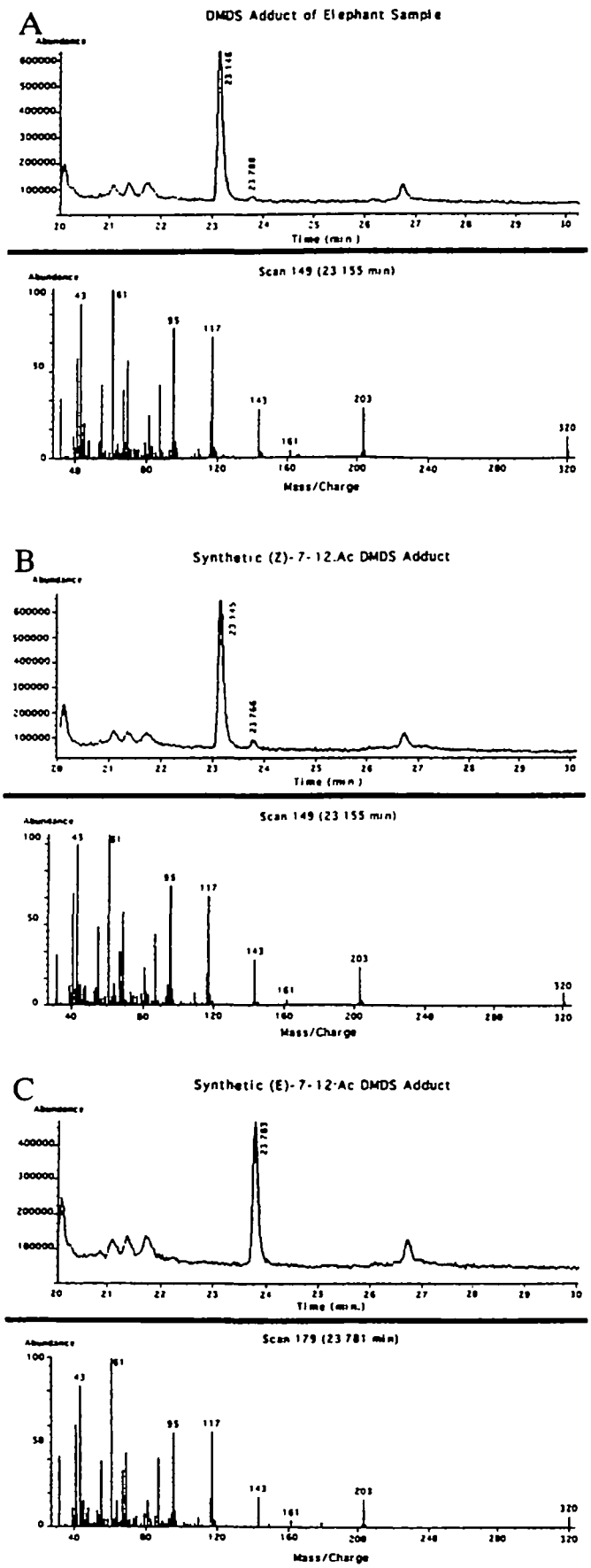


Figure 5 **(A)** The 500 MHz ^{1}H-NMR spectrum of the elephant pheromone, which is indicative of the (*Z*)-7-dodecen-1-yl acetate structure **(B)** The 500 MHz proton NMR spectrum of active elephant fraction; top is elephant pheromone sample; below is Z7-12·Ac standard



dodecadiene, except that the intensity of the molecular ion was greater than expected. It was reasonable to assume that some portion of the molecule was lost and the ion at *m/z* 166 was a fragment.

The NMR spectrum of the active fraction (Figure 5A) clearly established the structure to be dodecen-1-yl acetate. The multiplet at δ 5.4 (2H) indicates a di-substituted double bond, the triplet at δ 4.07 (2H) corresponds to a methylene attached to oxygen, and the broad singlet at δ 2.07 (4H) is consistent with methylene groups on either side of a di-substituted double bond. The singlet at δ 2.05 (3H) is consistent with an acetate. The NMR spectrum of a commercial sample of (*Z*)-7-dodecen-1-yl acetate was indistinguishable from that of the pheromone, as was the proton-decoupled ^{13}C-NMR spectrum (Figure 5B).

DMDS derivatization of the elephant pheromone compound and its comparison by capillary GC–MS to standards of *Z*- and *E*7-12:Ac resolved the position location of the single double bond, established the *Z* configuration for the major isomer and confirmed the molecular weight as 226. The mass spectrum of the major DMDS adduct derived from the pheromone exhibited a molecular ion at *m/z* 320 (226 plus the molecular weight of DMDS, 94) (Figure 6). DMDS derivatization of the pheromone produced two adducts and each produced a mass spectrum identical to that of the adducts of the synthetic *Z*- and *E*7-12:Ac, with diagnostic sulfide fragments at *m/z* 117 and 203 (Figure 6A). The GC retention time of the major adduct (97%) at 23.30 min was identical to that of the synthetic *Z*7-12:Ac adduct (Figure 6B), and the retention time of the minor adduct (3%) at 23.79 min was identical to that of the synthetic *E*7-12:Ac (Figure 6C). Therefore, the elephant pheromone as isolated was comprised of *Z*7-12:Ac (97%) and *E*7-12:Ac (3%). Whether this 3% *E* is produced naturally or as an artifact during the purification procedure is debated in the Discussion.

Figure 6 GC–MS of elephant pheromone **(A)**, compared with synthetic *Z*-(7) DMDS adduct **(B)**, and synthetic *E*-(7) DMDS adduct **(C)** A comparison of retention times with those of synthetic standards showed that the larger adduct peak (97%) at 23.15 was the *Z* isomer, and the smaller one at 23.78 (3%) was the *E* isomer The mass spectra of the DMDS adducts of elephant-derived (*Z*)-7-dodecen-1-yl acetate (A, bottom) were identical to those produced by synthetic *Z*7- (B, bottom) and *E*7-12:Ac (C, bottom) A comparison of retention times of the synthetic standards with those of the adducts showed that the larger adduct peak at 23 15 was the *Z* isomer (B) and the smaller one at 23.78 was the *E* isomer (C) The initial assay of the elephant pheromone was 97% of *Z*7-12.Ac and 3% of *E*7-12.Ac.

Table 2 Average concentration of Z7-12 Ac throughout estrous cycle of female Asian elephants (μg/ml)

	Luteal	Early follicular	Mid-follicular	Pre-ovulatory
Serum progesterone (ng/ml)[a]	400	175	76	35
Whole urine	nd[b]	0.48 (0 002 mM)	13.02 (0 058 mM)	33.0(0.146 mM)
Organic extracts				
Hexane				0.05 (0.0002 mM)
Dichloromethane				0.50 (0.002 mM)

[a]Serum progesterone concentrations are normally expressed as mass/volume

[b]Not detected

Quantitation of urinary Z7-12:Ac during the estrous cycle

The average concentration of Z7-12:Ac as determined by SPME, followed by GC–MS, changed during the estrous cycle as assessed at four discrete times: luteal (not detected), early follicular (0.002 mM or 0.48 μg/ml), mid-follicular (0.058 mM or 13 μg/ml), and just prior to ovulation (0.146 μM or 33 μg/ml) (Table 2). Concurrent serum progesterone levels are indicated in Table 2. Analysis of hexane and dichloromethane extracts indicated that most of the Z7-12:Ac chemical was lost in the extraction process (Table 2). Although no *E* isomer was detected during these methodologies, this isomer may be below the detection limit at some of the observed concentrations of *Z*.

Characterization of Z7-12:Ac in headspace of pre-ovulatory urine

A late eluting peak was observed in the volatile headspace compounds from female pre-ovulatory urine (Figure 7A). A similar peak was not observed with urine from the luteal phase. The mass spectrum of this peak (Figure 7B) was identical to the spectrum for the purified active elephant fraction obtained in Figure 4C, suggesting that it also was a fragment ion of Z7-12:Ac.

Synthetic Z7-12:Ac bioassay results

After the structure of the isolate was determined, bioassays of authentic synthetic Z7-12:Ac [both Z7-12:Ac containing traces (~2.5%) of the *E* isomer (Experiments 1, 2, 4 and 5) and ultrapure *E* or Z7-12:Ac (Experiment 3)] were conducted to establish that this compound was indeed the valid pheromone. Nine solitary captive Asian male elephants (and controls) in the US were assayed with up to 35 successive tests each (Tables 3–5). Sixteen male Asian elephants in the lumber camps of central Myanmar were assayed one to three times (Table 6).

The results with commercial Z7-12:Ac clearly confirmed its bioactivity. In every bioassay, although inter-assay variability occurred, a robust positive response was obtained.

The three trials with the pure isomers, 100% *Z* and 100% *E* at 1.0 mM concentrations, demonstrated no bioresponses by the five tested males to the *E* isomer, whereas the pure *Z* was bioactive (Table 5). Although the initial data suggest that the pure *Z* isomer is more active than the *Z* with traces (~2.5%) of E, we have not conducted sufficient tests to establish this trend firmly.

In Experiment 1 (Table 3), we compared male Asian elephant bioresponses elicited by urine-derived Z7-12:Ac and synthetic Z7-12:Ac. In addition, various concentrations of Z7-12:Ac were evaluated for their efficacy (Table 3). The chemosensory flehmen responses by Asian male elephants to Z7-12:Ac were compared statistically at several concentrations to a synthetic control (Table 3). The results demonstrate that responses by Asian male elephants to Z7-12:Ac were definitively and quantitatively higher across the spectrum of responses (Table 3) than were the responses to other synthetics identified from purified pre-ovulatory urinary fractions or the solvents used in extraction and bioassay. Flehmen responses, which are the clearest indicator of subsequent pre-mating and mating responses, were not different between authentic synthetic and elephant-derived Z7-12:Ac, although presentation in anestrous urine quantitatively increased responses (see Discussion). Flehmen responses by males nos 1, 2, 4 and 5 toward 2.0 and 5.0 mM concentrations of Z7-12:Ac were significantly higher than responses to controls (Table 3). Males nos 2 and 5 demonstrated significantly higher responses to 0.5 mM Z7-12:Ac than controls. Males nos 2–9 all demonstrated statistically significant elevations at 1.0

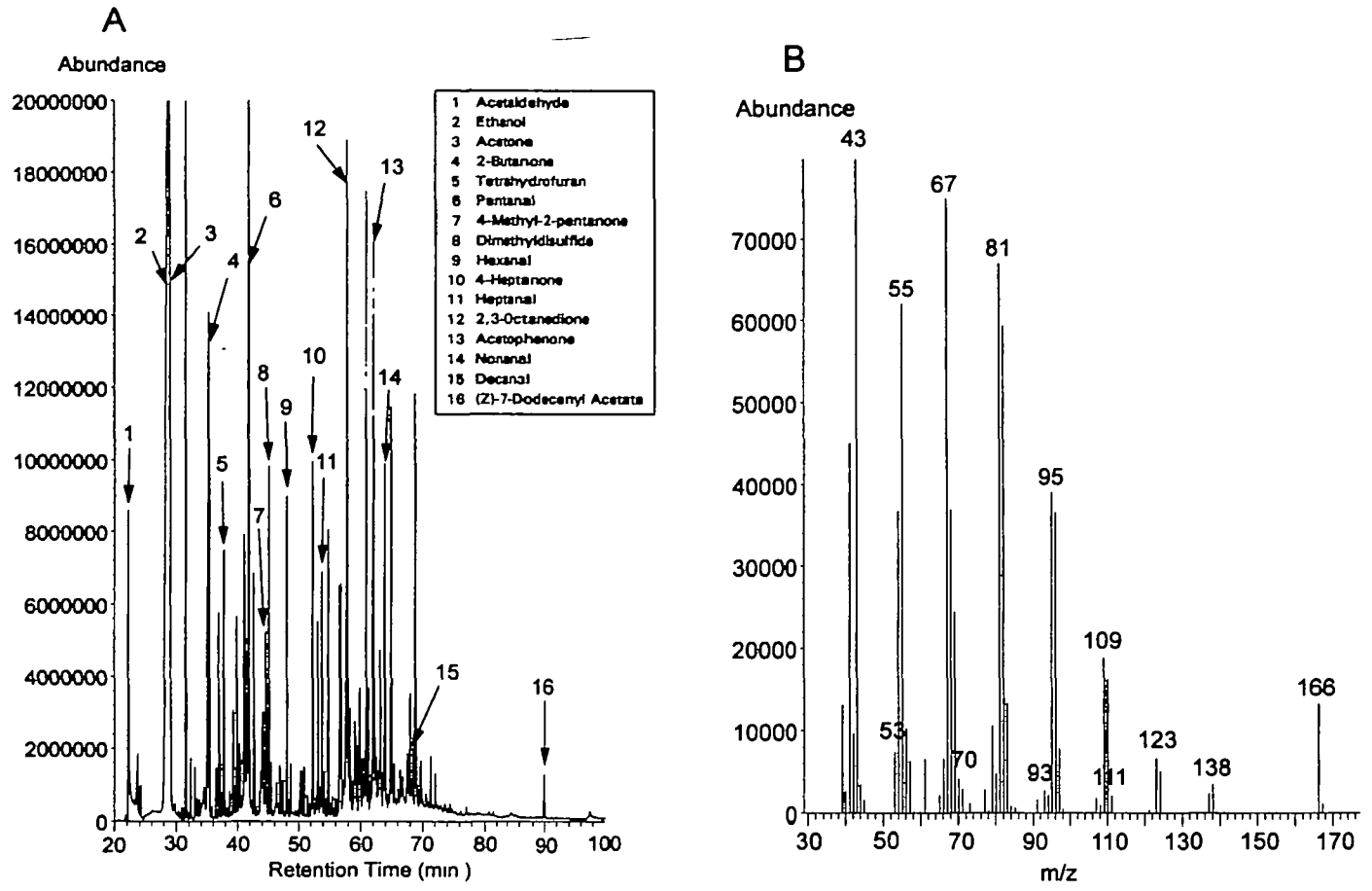


Figure 7 **(A)** GC–MS total ion chromatogram of the headspace of female pre-ovulatory urine, assessed bioactive by responses of male Asian elephants Fifteen relatively common volatiles in female urinary headspace are indicated, these ranged from ~50 to 500 ppbv *Z*7-12:Ac (peak 16) was detected at a late retention time **(B)** GC–MS spectrum of the fragment ion of the late-eluting *Z*7-12:Ac

mM *Z*7-12:Ac compared with controls (male no. 1 was the only exception). The conspecific and heterospecific controls demonstrated no flehmen responses with the exception of one African male elephant (Table 3). Flehmen responses were always significantly lower in African males and Asian females than recorded in male Asian elephants (Table 3).

Although the majority of the assays with synthetic *Z*7-12:Ac were significantly higher than control or solvent assays, they were lower than assays of whole urine or urine-extracted *Z*7-12:Ac (Table 3). Figure 9 presents data on the addition of *Z*7-12:Ac to control luteal phase anestrous urine.

In Experiment 2, male elephants nos 1–4 (Table 4) demonstrated no habituation to the synthetic version of *Z*7-12:Ac during these ten successive bioassays; the results from trials 1–3 were similar to those of trials 4–10 (Table 4). In contrast, all of the aldehyde controls, except dodecanal, elicited no responses after trial 3 (Table 4).

The flehmen response data from a single Asian male elephant (29 years old, experienced breeder) was plotted per bioassay for 15 successive assays during a 9 month period (Figure 8). Interassay variability was evident, but there were no statistically significant decreases in the frequency of flehmen responses with repetitive testing (Figure 8). Using Kruskal–Wallis one-way ANOVA on ranks, no differences were observed among frequencies of flehmen responses.

In Experiment 3, factors influencing bioresponse other than concentration were assessed, including pH of the presenting media and employment of non-pre-ovulatory urine as the presenting media. Figure 9 depicts three successive bioassays with four male Asian elephants of 1.0 mM *Z*7-12:Ac in 0.1 M acetone with acetate buffer (pH 6.0) as the presentation media. For all males, the presentation in mildly acidic buffers increased the chemoresponses, especially flehmen responses (Figure 9). For example, the flehmen response data for male no. 4 demonstrated significantly higher flehmen responses when *Z*7-12:Ac was suspended in buffer compared in water, $t = 3.2$, $P < 0.031$.

Table 3 Flehmen responses of solitary male Asian elephants during bioassays of authentic synthetic *Z*7-12:Ac

Test elephants	*Z*7-12 Ac concentration (mM)	Mean flehmen responses/h, 7 trials ± SE
Male no. 1	0.5	0 43 ± 0 20
	1.0	0 43 ± 0 20
	2.0	1 86 ± 0 40[a]*
	5.0	1 57 ± 0 42[a]*
Male no. 2	0.5	1 14 ± 0 55*
	1.0	1 28 ± 0 42[a]*
	2.0	1.86 ± 0.40[a]*
	5.0	1.71 ± 0 28*
Male no. 3	0 5	0.29 ± 0 18
	1 0	1.00 ± 0.37[a]*
	2 0	0 71 ± 0.29
Male no 4	0.5	0 29 ± 0 18
	1.0	2 29 ± 0 6*
	2.0	2 43 ± 0 48*
	5.0	2.14 ± 0.51*
Male no 5	0.5	2 60 ± 0 88[a]*
	1.0	1 50 ± 0 50*
Male no. 6	1 0	2.88 ± 1 14[a]*
Male no 7	1.0	3.00 ± 0 82[a]*
Male no 8	1.0	1.88 ± 0 61*
Male no 9	1.0	1.50 ± 0.87*
Conspecific and hetero-specific controls:		
African[b]	1.0	0.50 ± 0.29
Aldehydes[c]		
Hexanal		0.28 ± 0.09
Nonanal		0 57 ± 0.08
Decanal		0 29 ± 0 18
Dodecanal		0.17 ± 0 17
W-pre-O urine5[d]		7.00 ± 0 49
PEF[e]		5.00 ± 0.49

*Significant elevations (P = 0 05) in comparison with controls using one-way ANOVA on ranks, repeated measures When normality test failed, non-parametric tests were used because assumptions for parametric tests were violated Mann–Whitney rank sum test used, P = 0 001

[a]Erections observed

[b]The conspecific and heterospecific controls were three Asian female elephants and three African males Two males were housed together at the Riddle Elephant Sanctuary and the third was housed solitary at Ringling Center for Conservation. No flehmen responses were elicited by *Z*7-12 Ac from the three Asian females and the two co-housed African males Data for the third male is shown in the table.

[c]No flehmens were observed to the aldehydes, heptanal, octanal and undecanal, or to the solvents dichloromethane, acetone and acetonitrile

[d]Whole pre-ovulatory urine (500 ml)

[e]Positive elephant fraction.

Tests with six additional solitary males (not depicted) also demonstrated increased flehmen responses to *Z*7-12:Ac in buffer in comparison to water. Significantly increased bioresponses to *Z*7-12:Ac were also observed when anestrous urine was compared with either buffer or water as a presenting media (Figure 9).

In Experiment 4, during assays in Myanmar, seven of the 16 males were tested three times (Table 6A), five males were tested twice (Table 6B) and four males were tested once (Table 6C) with 1.0 mM *Z*7-12:Ac. Except for one instance, all males were tested in the presence of other males, with one

Table 4 Flehmen responses of four solitary male Asian elephants to compounds identified in purified pre-ovulatory urine fractions (mean responses/h ± SEM)

Substance	Trials 1–3	Trials 4–10
Synthetic *Z*7-12:Ac (2.0 mM)		
Male no 1	2.54 ± 0.57	2.51 ± 0.49
Male no 2	1.99 ± 0 45	1.60 ± 0.55
Male no. 3	1.00 ± 0.37	1.25 ± 0.11
Male no. 4	2.43 ± 0.48	2.10 ± 0.33
Elephant preparation controls		
Whole pre-ovulatory urine[a]	7.00 ± 0 49	7.00 ± 0.49
Positive elephant fraction[a]	5.00 ± 0 49	5.00 ± 0.49
Synthetic control		
Novel substance· 10 mM		
2-n-propylphenol	4.00 ± 0.57[b1]	0
Aldehydes (identified by GC–MS) in late-stage active fraction (1 mM)[c]		
Hexanal	0 28 ± 0.09[b2]	0
Nonanal	0.57 ± 0.08[b3]	0
Decanal	0.29 ± 0.18[b4]	0
Dodecanal	0.17 ± 0.17	0 14 ± 0 14

[a]500 ml pre-ovulatory urine or equivalent.

[b]Paired *t*-test for statistical differences between groups Normality failed, Mann–Whitney rank sum. Data were converted to medians (25th–75th percentiles) **(1)** 4 00 (3.25–4.75), T = 28, P < 0 001, **(2)** 0 24 (0 14–0 38) T = 6, P = 0 05, **(3)** 0 60 (0 45–0.68), T = 6, P = 0.05, **(4)** 0 25 (0 14–0 39), T = 6, P = 0 05

[c]No flehmens were observed to the negative control synthetic 4-n-propylphenol (10 mM), the aldehydes, heptanal, octanal or undecanal, even at the first trial. No flehmens were observed for up to 0 6 M solvent controls (acetone, acetonitrile and dichloromethane) during any test

Table 5 Mean flehmen responses (± SE) of five mature solitary male Asian elephants

Test substance	Flehmen responses (mean ± SE /h)
Pure *Z* isomer (1.0 mM)	5 00 ± 0.49*
Pure E isomer (1 0 mM)	0.50 ± 0.29
*Z*7-12 Ac purified from estrous urine	5 00 ± 0.49*
Pre-ovulatory urine	7 00 ± 0.49*

*These flehmen responses/h were statistically significantly elevated over the responses to the pure *E* isomer. One-way ANOVA on ranks, repeated measures

Table 6 Non-solitary male Asian elephants tested with 1 mM Z7-12 Ac in Myanmar lumber camps (in comparison with acetone control)

Triple-tested = A		Twice-tested = B		Single-tested = C
Male no.1*		Male no 8		Male no 9
Sniffs[a]		1	1	
Checks[b]	28.67 ± 15.90			12
Places[c]	4 00 ± 1.53	3		
Flehmens	6.33 ± 3 18	1		1
Backs up[d]		1	1	
Male no.2***		Male no 10		Male no.14*
Sniffs		1	2	1
Checks	60.33 ± 14.95	1	2	
Places	8 97 ± 1 20			
Flehmens	10 00 ± 5 30			
Backs up		1	3	1
Male no.3***		Male no 11		Male no 15
Sniffs		1	3	5
Checks	8 33 ± 2 03			
Places	2.33 ± 0.69			
Flehmens	2.33 ± 1 33		4[e]	
Backs up		3		
Male no 4**		Male no.12		Male no.16
Sniffs		6	1	
Checks	15.33 ± 6 57			
Places	2.67 ± 0 88			
Flehmens	2.33 ± 1.33	1	1	2
Backs up		3	2	
Male no.5		Male no.13		
Sniffs		3	2	
Checks	6.33 ± 2.40		1	
Places	2.33 ± 0.33			
Flehmens	3.00 ± 1.52			
Backs up		2	1	
Male no.6				
Checks	6.67 ± 2.19			
Places	5 00 ± 2.52			
Flehmens	2.68 ± 1.20			
Male no.7				
Checks	2.33 ± 0.88			
Places	1.67 ± 1.20			
Flehmens	3 00 ± 1.00			
Males no.1–7 0.1 M acetone control				
Checks	1 25 ± 0.50			
Places	0.75 ± 0.20			
Flehmens	0.00 ± 0.00			

Data expressed for triple-tested males as mean of three tests

*Number of erections demonstrated.

[a]Sniff responses are audible olfactory responses [b]Check responses occur when the trunk tip finger is distinctly placed in contact with liquid sample (see Introduction) [c]Place responses occur when the entire trunk tip is placed in contact with the sample These responses occur independently or can occur prior to and as an integral part of the flehmen. [d]Back up responses are retreats from area of sample, often before touching sample [e]Later exhibited chemosensory responses as solitary animal

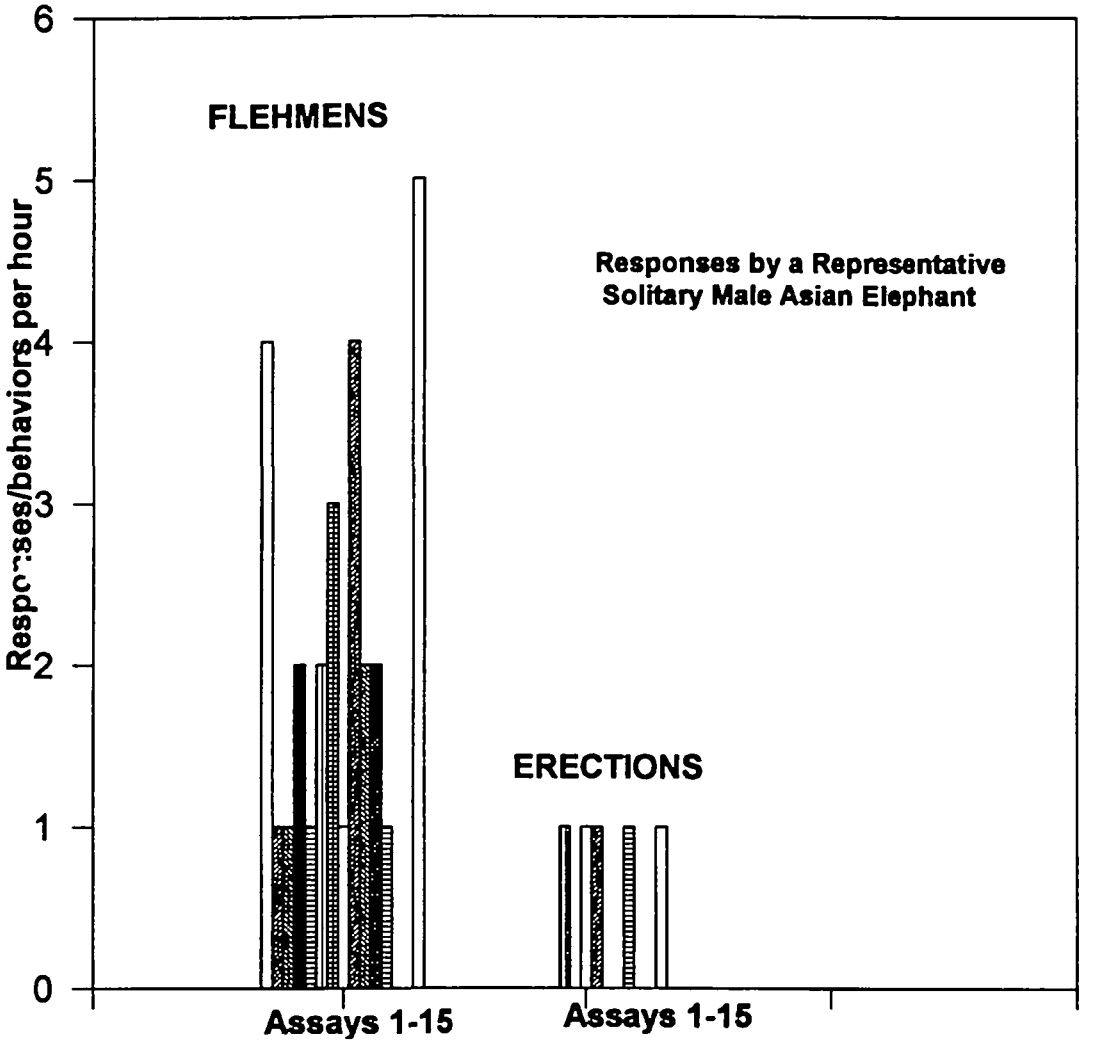


Figure 8 Fifteen successive bioassays of 0 5 mM *Z*7-12 Ac with a representative mature male Asian elephant Flehmen responses/h per bioassay and the number of erections, a physiological behavioral response, are depicted

male at a time allowed access to the sample. Males nos 1–7 were triple-tested with 1.0 mM *Z*7-12:Ac and demonstrated chemosensory responses somewhat higher quantitatively (at times significantly higher) than those observed by solitary males tested in the USA. Mean flehmen responses to 1.0 mM *Z*7-12:Ac varied between 2.33 and 10.00 responses/h by male elephants in Myanmar and between 0.43 and 3.00 responses/h by solitary males tested in the USA. However, four of the five males tested only twice, and two of the four males tested only once, exhibited either mild interest (sniffs) or avoidance (blows, trunk shakes, walking away) (Table 6). Male no. 11 in the twice-tested group only sniffed and backed up from the *Z*7-12:Ac on the first test; however, when retested as a solitary animal, this same male responded with four flehmens.

These results clearly indicate that male Asian elephants respond in a non-habituating fashion to synthetic *Z*7-12:Ac. A threshold concentration is apparent, between 0.5 and 1.0 mM concentration, but a dose–response effect has not been observed. Males exhibit considerable inter-animal response variation and some inter-assay response variation, but the responses to *Z*7-12:Ac are significantly above responses to controls. The results from control animals (Asian females and African males) demonstrated significantly lower flehmen responses than the Asian male elephants. In addition, erections were observed in 22% of the assays with the nine solitary Asian male elephants in the US. In Myanmar none of the twice-tested males demonstrated erections and all showed some degree of backing up. However, with the non-solitary males that responded positively in three assays, erections were observed in 43% of tests.

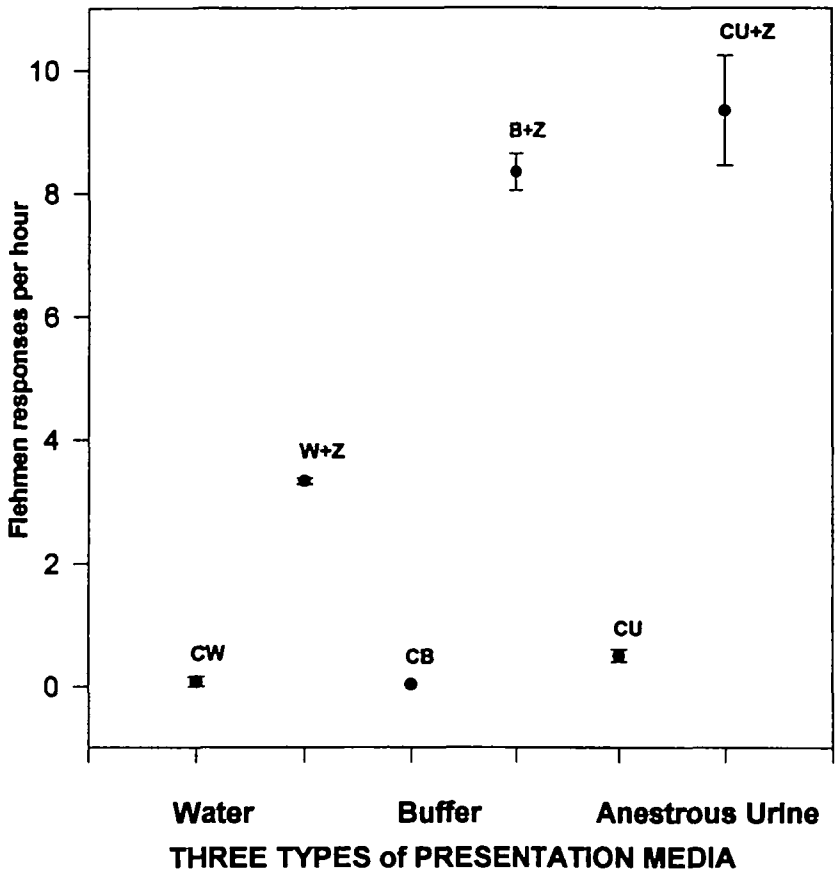


Figure 9 Quantitative differentials in flehmen responses/hour during three successive assays (mean ± SE) of 1 0 mM *Z*7-12 Ac in three types of presentation media: water, mildly acidic acetate buffer (0 2 M, pH 6 0), and control anestrous urine. Control samples without *Z*7-12 Ac were water (W), buffer (B) and anestrous urine (CU) Paired assay samples with 1 0 mM *Z*7-12·Ac were in water (W+Z), in buffer (B+Z) and in anestrous urine (CU+Z)

Discussion

The isolation of a highly bioactive fraction from the chemically complex urine of female elephants in estrus and its purification as a single component was an arduous task. Essential to the purification process was a reliable quantitative bioassay that guided the chemical fractionation. The organic extracts were extremely complex mixtures with high concentrations of some phenolic components. Aqueous sodium hydroxide (2%) partitioning effectively removed much inactive material without depleting bioactivity. Subsequently, concurrent GC analyses assessed purity during final stages of HPLC purification, with GC–MS providing the identification of minor components within the active preparation. Finally, GC–MS and NMR suggested a dodecenyl acetate, and a DMDS adduct technique was employed to establish the *Z* isomer and double-bond positioning.

The presence of the active chemosignal in the complex urinary excretion made its isolation and purification unusually difficult. Hexane extraction of *Z*7-12:Ac was 10-fold lower in yield than that of dichloromethane extraction. In contrast, *Z*7-12:Ac is readily extracted by hexane from insect gland secretions, which often have a less complex chemical composition. Dichloromethane extracted *Z*7-12:Ac from elephant urine with retention of high bioactivity (Rasmussen *et al.*, 1982), yet the yield was very low (1–2%). Possibly, the extractability of this acetate may be affected by binding to protein carriers. One focus of our current investigations is on the characterization of urinary proteins that may bind to the *Z*7-12:Ac pheromone and may confer specificity. This possibility is suggested by the demonstrated binding of *Z*5-12:Ac and its fluorinated analogues to proteins in insects (Prestwich *et al.*, 1995) and by preliminary binding evidence with elephant urine (L.E.L. Rasmussen and G.D. Prestwich, unpublished data).

Among invertebrates, including insects such as Lepidoptera, the active sex pheromones are often specific ratios of closely related, often hydrocarbon species (Roelofs, 1995), and the exquisite selectivity of pheromone specificity is

encoded in the protein components of the male antennal sensillum lymph and dendritic membrane (Prestwich *et al.*, 1995; Roelofs, 1995). Such protein components decode information encrypted in the blends of volatile odorants produced by female moths (Vogt *et al.*, 1991a,b; Prestwich *et al*, 1995). While hundreds of insect pheromones and pheromone blends have been identified (Mayer and McLaughlin, 1991), identification of sex pheromones is rare among vertebrate classes. An exception is the well-described sex pheromonal system among fishes. Specificity appears to be conferred by precise ratios, often differing by minute percentages of closely related hormonal chemical species, such as prostaglandins and steroid hormones and their metabolites, especially substituted progesterones (Sorensen and Goetz, 1993; Sorensen and Scott, 1994). The more evolutionarily advanced snakes, such as the Canadian red-sided garter snake (*Thamnophis sirtalis parietalis*), use a series of skin-derived long-chain methyl ketones (Mason *et al.*, 1989). Interestingly, several acetates have been described in mammals to have attractant and/or pheromone-like properties. Some of these active compounds are saturated alkyl acetates. For example, decyl propionate from the preputial gland of infant rats elicits maternal anogenital licking (Brouette-Lahlou *et al.*, 1991). Female rats prefer the odor of a specific fraction of male rat preputial gland (Gawienowski *et al.*, 1975; Stacewicz-Sapuntzakis *et al.*, 1977) and especially certain aliphatic acetates, including saturated C12 acetates (Gawienowski *et al.*, 1977). Novel, species-typical esters have also been described from the preputial glands of two sympatric voles (Welsch *et al.*, 1988).

Little knowledge exists regarding the chemical nature of mating-associated pheromones for mammals considered to be of high intelligence, e.g. those with enlarged and convoluted cerebral cortexes, complex, multi-level communication systems, creative use of a repertoire of tools and an organized sophisticated society. Our identification of *Z*7-12:Ac as a sex pheromone of the Asian elephant offers some comparisons with known mammalian sex pheromones. Among several rodents, including hamsters, and the domestic pig, specific releaser sex pheromones have been chemically and structurally identified and linked to specific sexual behaviors (Singer *et al.*, 1976; Jemiolo *et al.*, 1985; Novotny *et al.*, 1985, 1986; Schwende *et al.*, 1986; Dorries *et al.*, 1995). Male pigs release two steroids, androstenone (5α-androst-16-ene-3-one) and androstenol (5α-androst-16-en-3β-ol), in their saliva that elicit lordosis by females. Dimethyl disulfide has been characterized as a female-to-male sex attractant (Singer *et al.*, 1976, 1986), although recent evidence suggests it is a general attractant because females as well as males respond to this substance (Johnston and Petrulis, 1994). Aphrodisin, a protein isolated from female hamster vaginal secretions, stimulates copulatory behavior in males (Singer *et al.*, 1984a,b, 1986, 1988). Subsequent studies by Singer on hamster and mouse pheromones indicate that a protein–ligand complex, or perhaps just the ligand, is essential for biological activity (Singer, 1991). Our identification of *Z*7-12:Ac functioning as a sex pheromone will allow its study in comparison with these two known mammalian sexual pheromonal systems. *Z*7-12:Ac offers a distinct advantage as its physical and chemical properties and biological activity, including signal transduction, have been studied for a number of years in insects (Prestwich *et al.*, 1995; Roelofs, 1995). Relevant questions concerning possible urinary protein carriers similar to the major urinary proteins of mice (Robertson *et al.*, 1996), specific mucous proteins, and the mechanism of signal transduction in this unusual, yet potentially model mammal can now be more readily addressed.

Several fundamental questions arise following the elucidation of the chemical identity of the pre-ovulatory pheromone. Knowledge of the specific chemical compound that is released in the urine by the about-to-ovulate female Asian elephant will allow specific behaviors (or physiological reactions) by conspecific male elephants to be defined. We have known for many years that pre-ovulatory urine evoked several specific chemosensory responses, followed by a number of pre-mating and mating behavioral responses. Our data in this current paper and in previous studies (Rasmussen *et al.*, 1982, 1986, 1993) suggest that the primary activity of *Z*7-12:Ac is as a releaser pheromone, with activity as (i) an attractant pheromone and (ii) a precopulatory pheromone. Now it will be possible to establish, through behavioral, physiological and endocrinological studies, the extent of the attractive and copulatory actions of *Z*7-12:Ac as a releaser pheromone and whether it has any effects as a (iii) long-term primer pheromone.

With the identity of the pheromone known, we have demonstrated *Z*7-12:Ac as a minor component in the headspace volatiles of pre-ovulatory urine. We have made the initial assessments of concentrations in the native female urine during the estrous cycle. In addition, we have initial data on the percent yield during the initial organic solvent extraction. The percent yield was very low, ~0.2% for the hexane extract, which was not used during the purification

procedure as bioactivity was not elicited, and 1–2% for the dicloromethane extract. However, recent analyses of urine using the SPME technique indicate much higher pheromone concentrations than from the low-yield solvent extractions. Initial bioassay results with the synthetic *Z*7-12:Ac demonstrated more inter-male and inter-assay variability (at identical concentrations) than was demonstrated with the native extracted *Z*7-12:Ac. This variability suggests that threshold levels may be reached, that higher concentrations do not increase responses through possible saturation of receptors, that other components are required for precise activity, or that mating experience and physiological state play a significant role in response intensities. For example, an absolute dependence of response intensity on concentration was not apparent. In addition, 0.5 mM or higher concentrations of synthetic *Z*7-12:Ac as presented in water were required consistently to elicit multiple flehmen responses in male elephants. (Only one of the several tests conducted at 0.1 mM concentration elicited high levels of bioresponses.) The 0.5 mM or higher concentrations required for consistent elicitation of bioactivity were higher than the (0.146 mM) concentration measured in bioactive, native pre-ovulatory urine. However, presentations of 1.0 mM *Z*7-12:Ac in control urine elicited elevated bioresponses compared with presentation in water or buffer (Figure 9). In addition, several tests between 0.1 and 0.5 mM in control urine demonstrated again elevated bioresponses. These were equivalent to bioresponses elicited by pre-ovulatory urine containing similar concentrations of native *Z*7-12:Ac. Substances in urine, perhaps proteinaceous, may affect the final bioactivity and partially explain the differences between the bioactivity of the pure *Z*7-12:Ac presented in water as compared with control urine.

Behavioral responsiveness to pheromones is presumably instinctual, but can be influenced by mating experience, physiological state, or social context. At one assay site in the USA, a male was observed repetitively comparing *Z*7-12:Ac with other samples and to actual females. At times, naive males may attempt to mate anestrous females or even logs. Only further contextual studies will address such questions as how powerfully an elephantine sex pheromone can override experience, physiological state, or social status.

One of the most remarkable aspects of this study is just emerging. Table 6 shows the results from assays of *Z*7-12:Ac with male Asian elephants in the lumber camps of Myanmar. All the previous assays of pre-ovulatory urine, its extracts and fractions, and the synthetic identified *Z*7-12:Ac were conducted on solitary Asian male elephants within the USA. All of these US males were housed in facilities with other males, but none were allowed physical contact with their conspecifics. In Myanmar, all tests were conducted with multiple males present.

The dominance status of the males in multiple groups apparently affected their chemosensory responses to a female pheromone. In Myanmar, excellent written records and personal observations by oozies (Burmese rider-handlers of elephants, who have life-long care and management of particular elephants) make it possible to assess in a relative manner the dominance position and access to estrous females of particular males. This information was available for the males tested in this report. Although detailed, long-term observations were not possible by the investigator, clear indications of dominance and subdominance (Rasmussen, 1988) were indicated prior to, during and after the test periods. Fascinatingly, the examination of the social status of the tested males revealed that all the responder males were dominant animals who either had ready access to females or were known to be proven breeders. Conversely, all the animals that either backed away from the sample or ignored it were the younger subdominant males. Clearly, the context and the societal structure of the elephant needs to be considered carefully in further studies of *Z*7-12:Ac as a sex pheromone. The importance of social context is reinforced by comparison with earlier studies of rat preputial gland secretions that demonstrated hexadecyl acetate in the marking urine. Dominant males marked more frequently in response to hexadecyl acetate-enriched urine than to urine alone; the opposite reaction was shown by subordinate males (Brinck and Hoffmeyer, 1984).

Some additional fundamental questions remain: (i) What is the role, if any, of the small percentage of *E* isomers? Is the *E* isomer, detected in the material isolated from the elephants, present originally or formed by isomerization during the multi-stepped purification? (ii) How is the acetate synthesized? How does the elephant synthesize this acetate, as Lepidopterans possess a unique Δ-11 desaturatase (Bjostad and Roelofs, 1983; Roelofs and Wolf, 1988; Roelofs, 1995). Does the elephant have a *Z*7-desaturatase, and is it produced by a chain-shortening (*Z*)-9-tetradecanoyl precursor? What are the chain-shortening mechanisms? (iii) Where is the acetate synthesized in the elephant? Is there a bacterial contribution? It is possible that the pheromone might be derived from an organism living in the urogenital system, perhaps triggered to develop as a

function of the endocrine environment? (iv) What are the implications for co-evolution of this acetate in widely separated species? Does elephant urine containing this pheromone affect Lepidopteran species living in the locale? The lack of reports of moths being attracted to elephant urine suggests that the great complexity of compounds in the mammalian urine may mask the effect of Z7-12:Ac for insects, in addition to the use of blends by insects.

It is remarkable that the female Asian elephant employs, as her pre-ovulatory sex awareness and arousal pheromone, the identical compound that has been found as a component in attractant blends for about 140 Lepidopteran species in 13 families (Arn *et al.*, 1992). These species all utilize (Z)-7-dodecen-1-yl acetate as an essential component of their female-to-male sex pheromone attraction blend That (Z)-7-dodecen-1-yl acetate is the major component of the Asian elephant urinary sex pheromone is indeed an unexpected finding of fundamental importance to the field of mammalian chemical communication.

ACKNOWLEDGEMENTS

We thank the Metro Washington Park Zoo, especially Dr Michael Schmidt, Research Veterinarian, and Mr Roger Henneous, Senior Elephant Keeper, for their unstinting aid during the long course of this project; additional thanks are extended to Mr Fred Marion, Ms Sioux Marion, Mr Charles Rutkowski, Mr Jay Haight, Mr Ray Hopper, Dr Jill Mellen and Director Sherry Sheng. This study was funded in part by grant RO1 HD19219 from the National Institutes of Health and by a 1993 Guggenheim Fellowship to L.E.L. Rasmussen. Additional support was provided by the Eppley Foundation.

REFERENCES

Altmann, J. (1974) Observational study of behavior sampling methods *Behaviour*, **49**, 227–265.

Arn, H., Toth, M and Priesner, E (1992) *List of Sex Pheromones of Lepidoptera and Related Attractants* International Organization for Biological Control, Montfavet, France Also web site http://www nysaes cornell edu/pheronet/

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

Brinck, C and Hoffmeyer, I. (1984) Marking urine and preputial gland secretion of male bank voles *J Chem Ecol*, **10**, 1295–1307

Brouette-Lahlou, I , Amouroux, R , Chastrette, F, Cosnier, J , Stoffelsma, J. and Vernet-Maury, E. (1991) Dodecyl propionate, attractant from rat pup preputial gland. characterization and identification. *J. Chem. Ecol.*, **17**, 1343–1354.

Buser, H.-R., Arn, H , Guerin, P and Rauscher, S (1983) Determination of double bond position in mono-unsaturated acetates by mass spectrometry of dimethyl disulfide adducts *Anal Chem.*, **55**, 818–822.

Buss, I.O (1990) *Elephant Life Fifteen Years of High Population Density* Iowa State University Press, Ames, IA

Dorries, K M , Adkins-Regan, E and Halpern, B.P (1995) Olfactory sensitivity to the pheromone, androstenone, is sexually dimorphic in the pig *Physiol Behav*, **57**, 255–259

Eisenberg, J.F., McKay, G M and Jainudeen, M R (1971) Reproductive behavior of the Asiatic elephant (Elephas maximus maximus L) *Behaviour*, **38**, 193–225

Gawienowski, A.M , Orsulak, P J , Stacewicz-Sapuntzakis, M and Joseph, B M (1975) Presence of sex pheromone in preputial glands of male rats. *J. Endocrinol*, **67**, 283–288.

Gawienowski, A M., Orsulak, P J , Stacewicz-Sapuntzakis, M. and Pratt, J J (1976) Attractant effect of female preputial gland extracts on the male rat *Psychoneuroendocrinology*, **1**, 411–418.

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

Jemiolo, B , Alberts, J., Sochinski-Wiggins, S., Harvey, S. and Novotny, M (1985) Behavioural and endocrine responses of female mice to synthetic analogues of volatile compounds in male urine. *Anim Behav.*, **33**, 1114–1118.

Johnston, R E and Petrulis, A. (1994) Re-evaluation of dimethyl disulfide as a sex attractant in golden hamsters. *International Society of Chemical Ecology, 11th Annual Meeting, June 4–8*, Syracuse, NY.

Mason, R.T., Fales, H.M., Jones, T.H., Pannell, L.K., Chinn, J.W. and

Crews, D. (1989) Sex pheromones in snakes *Science*, **245**, 290–293.

Mayer, M.S. and McLaughlin, J.R (1991) *Handbook of Insect Pheromones and Sex Attractants*. CRC Press, Boca Raton, FL

Novotny, M , Harvey, S , Jemiolo, B. and Alberts, J (1985) Synthetic pheromones that promote inter-male aggression in mice *Proc Natl Acad Sci USA*, **82**, 2059–2061

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* , **22**, 207–221.

Prestwich, G D., Du, G. and Laforest, S (1995) How is pheromone specificity encoded in proteins? *Chem. Senses*, **20**, 461–469.

Rasmussen, L E L. (1997) Chemical communication· an integral part of functional Asian elephant (*Elephas maximus*) society. *Ecoscience*, in press

Rasmussen, L.E.L and Hultgren, B. (1990) Gross and microscopic anatomy of the vomeronasal organ in the Asian elephant, Elephas maximus. In McDonald, D , Muller-Schwarze, D and Natynczuk, S.E (eds), *Chemical Signals in Vertebrates* Oxford University Press, Oxford, Vol 5, pp 154–161.

Rasmussen, L E.L. and Munger, B.L. (1996) The sensorineural specializations of the trunk tip (finger) of the Asian elephant, Elephas maximus *Anat Rec* , **246**, 127–134.

Rasmussen, L.E., 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 , Schmidt, M J and Daves, G.D. Jr (1986) Chemical communication among Asian elephants. In Duvall, D , Silverstein, M and Muller-Schwarze, D (eds), *Chemical Signals in Vertebrates· Evolutionary, Ecological, and Comparative Aspects*. Plenum Press, New York, Vol. 4, pp. 627–671

Rasmussen, L.E.L., Lee, T D , Daves, G D. Jr and Schmidt, M.J. (1993) Female-to-male pheromones of low volatility in the Asian elephant, *Elephas maximus J. Chem. Ecol.*, **19**, 2115–2128.

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

Robertson, D H.L., Cox, K.A., Gaskell, S.J., Evershed, R.P and Beynon, R.J. (1996) Molecular heterogeneity in the major urinary proteins of the house mouse *Mus musculus Biochem J.*, **316**, 265–272.

Roelofs, W.L. (1995) Chemistry of sex attraction. *Proc Natl Acad. Sci. USA*, **92**, 44–49.

Roelofs, W.L. and Brown, R L (1982) Pheromones and evolutionary relationships of Tortricidae. *Annu Rev Ecol. Syst* , **13**, 395–422

Roelofs, W.L. and Wolf, W.A (1988) Pheromone biosynthesis in Lepidoptera. *J Chem Ecol* , **14**, 2019–2031

Schwende, F.J., Wiesler, D , Jorgenson, J.W., Carmack, M and Novotny, M. (1986) Urinary volatile constituents of the house mouse, Mus musculus, and their endocrine dependency *J Chem Ecol.*, **12**, 227–296

Singer, A.G (1991) A chemistry of mammalian pheromones *J Steroid Biochem Mol Biol* , **39**, 627–632

Singer, A.G. and Macrides, F (1993) Composition of an aphrodisiac pheromone *Chem. Senses*, **18**, 630

Singer, A.G , Agosta, W.C., O'Connell, R J , Pfaffmann, C., Bowen, D.V. and Field, F.H. (1976) Dimethyl disulfide an attractant pheromone in hamster vaginal secretions. *Science*, **191**, 948–950

Singer, A G , Clancy, A N , Macrides, F. and Agosta, W C (1984a) Chemical studies of hamster vaginal discharge. effects of endocrine ablation and protein digestion on behaviorally active macromolecular fractions *Physiol Behav* , **33**, 639–643

Singer, A G., Clancy, A N , Macrides, F and Agosta, W.C (1984b) Chemical studies of hamster vaginal discharge. male behavioral responses to a high molecular weight fraction require physical contact. *Physiol. Behav* , **33**, 645–651.

Singer, A G., Macrides, F, Clancy, A N and Agosta, W C. (1986) Purification and analysis of a proteinaceous aphrodisiac pheromone from hamster vaginal discharge *J Biol Chem.*, **261**, 13323–13326

Singer, A.G., Clancy, A.N , Macrides, F., Agosta, W.C. and Bronson, F.H (1988) Chemical properties of a female mouse that simulate gonadotropin secretion in males *Biol Reprod.*, **38**, 193–199.

Sorensen, P W (1992a) Hormones, pheromones and chemoreception In Hara, T J (ed), *Fish Chemoreception* Chapman & Hall, London, pp. 199–228.

Sorensen, P W (1992b) Hormonally derived sex pheromones in goldfish, a model system for understanding the evolution of sex pheromone systems in fish. *Biol. Bull (Woods Hole)*, **183**, 173–177.

Sorensen, P.W. and Goetz, F.W. (1993) Pheromonal and reproductive function of F-prostaglandins and their metabolites in teleost fish. *J. Lipid Mediators*, **6**, 1–3

Sorensen, P.W. and Scott, A.P. (1994) The evolution of hormonal sex pheromones in teleost fish: poor correlation between the pattern of steroid release by goldfish and olfactory sensitivity suggests that these cues evolved as a result of chemical spying rather than signal specialization. *Acta Physiol. Scand* , **152**, 191–205.

Stacewicz-Sapuntzakis, M and Gawienowski, A.M. (1977) Rat olfactory response to aliphatic acetates. *J Chem. Ecol.*, **3**, 411–417

Still, W., Kahn, M and Mitra, A. (1978) Rapid chromatographic technique for preparative separation with moderate resolution. *J Org. Chem*, **43**, 2923

Sukumar, R (1989) *The Asian Elephant, Ecology and Management*. Cambridge University Press, Cambridge.

Vogt, R G, Prestwich, G D. and Lerner, M R. (1991a) Odorant binding protein subfamilies associate with distinct classes of olfactory receptor neurons in insects *J. Neurobiol*, **22**, 74–84.

Vogt, R G, Rybczynski, R. and Lerner, M R. (1991b) Molecular cloning and sequencing of general odorant-binding proteins GOBP1 and GOBP2 from the tobacco hawk moth Manduca sexta comparison with other insect OBPs and their signal peptides *J Neurosci*, **11**, 2972–2984.

Welsch, C.J., Moore, R E., Bartelt, R.J. and Jackson, L.L. (1988) Novel, species-typical esters from preputial glands of sympatric voles, *Microtus montanus* and *M pennsylvanicus*. *J. Chem. Ecol*, **14**, 143–158

Wysocki, C.J. (1979) Neurobehavioral evidence for the involvement of the vomeronasal system in mammalian reproduction. *Neurosci. Biobehav. Rev*, **3**, 301–341

Wysocki, C.J, Bean, N.J and Beauchamp, G.K. (1986) The mammalian vomeronasal system: its role in learning and social behavior. In Duvall, D. and Muller-Schwarze, D (eds), *Chemical Signals in Vertebrates* Plenum, New York, Vol. 4, pp. 471–483.

Received October 21, 1996, accepted April 28, 1997