Elephas maximus; MALE TO FEMALE CHEMOSIGNALS (PHEROMONES) FROM TEMPORAL GLAND SECRETION AND THEIR CHEMICAL ANALYSES

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The thesis "*Elephas maximus;* Male to Female Chemosignals (Pheromones) From Temporal Gland Secretion and Their Chemical Analyses" by Thomas E. Perrin has been examined and approved by the following Examination Committee:

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DEDICATION

I dedicate this work to my parents, Eugene and Marianne Perrin.

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ABSTRACT

Elephas maximus; Male to Female Chemosignals (Pheromones) from Temporal Gland Secretion and Their Chemical Analyses

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Male Asian elephants (*Elephas maximus*) exude an aqueous, odoriferous fluid from the temporal gland (located in the temporal fossa region) during their musth periods. This temporal gland secretion (TGS) elicits two distinct categories of behaviors in female Asian elephants. One category is trunk-to-mouth mediated behavior and includes the flehmen and palatal pit area contact responses. Flehmen and palatal pit area contact responses are often accompanied by two other trunk behaviors, scrub and check responses. Another category of female behavior associated with TGS, which is apparently olfactory mediated, includes avoiding reactions. Avoiding reactions involve instantaneous retreat (i.e., backing up) subsequent to presentation of TGS. Avoiding reactions are periodically accompanied by rapid ear movement and vocalizations (trumpeting, growling, and squeaking). Based on these observations, it was hypothesized that there are male-to-female chemical signals present in TGS that elicit these responses in the females. The goal of this study was to isolate and identify chemosignals in male musth TGS that elicited both trunk-to-mouth behavior and olfactory-mediated avoiding reactions in female Asian elephants.

Cyclohexanone, a naturally occurring component of male Asian elephant TGS, was tested as a candidate elicitor of trunk-to-mouth bioresponses among female Asian elephants. Cyclohexanone elicited persistent flehmen, palatal pit area contact, scrub, and check responses among a group of four female Asian elephants. The results suggested that cyclohexanone may provide chemical information to females about male elephants, particularly regarding their state of musth.

Avoiding reactions by the females were apparently caused by volatile compounds in the headspace of whole, fresh TGS. A method was developed to collect, store, analyze, and bioassay the volatile headspace compounds of TGS. Utilizing this method, a headspace fraction from TGS that elicited avoiding reactions among the females was isolated. The results showed that there were distinct chemical differences between biologically active, i.e., avoidance eliciting, and inactive TGS. Ten identified compounds were unique to the headspace of bioactive TGS and were apparently present in all bioactive TGS samples. This indicated that perhaps one or more of these ten compounds elicited avoiding reactions in the females.

Results of both studies supported the hypothesis that male-to-female chemical signals exist in male Asian elephant temporal gland secretion.

CHAPTER I

INTRODUCTION

MAMMALIAN CHEMICAL COMMUNICATION

Pheromone-mediated communication is vital for the regulation of mammalian social behavior. A pheromone is a compound or mixture of compounds utilized for inter-species communication. Pheromones are secreted in urine, vaginal discharges, sweat, feces, saliva, and other exudates. When received by another individual of the same species, pheromones elicit specific behavioral responses or physiological changes (Karlson & Luscher, 1959) related to, for example, reproduction, territorial establishment, individual identification, and maintenance of young-mother recognition.

The three primary chemosensory systems utilized for the reception of pheromones of mammals include olfaction, taste, and the vomeronasal organ. The anatomy, physiology, and function of these three primary pheromone reception systems are briefly described below.

Mammalian olfaction is mediated in the nasal cavity. The lateral walls of the nasal cavity are elaborated into a variable complex series of folds by bony turbinal extensions and covered with a highly vascularized mucosa membrane (Farbman, 1992). In the posterior olfactory chamber, much of the surface area of the turbinal extensions is lined with olfactory epithelium. In mammals with

a highly developed, keen sense of smell (e.g., dogs, deer, rodents, and elephants) these folds are highly convoluted and extended.

The process of mammalian olfactory chemoreception is described as follows. Compounds (chemosignals) that stimulate the olfactory system must be volatile, i.e., they must have a relatively high vapor pressure. Most odorous substances, including chemosignals, have a molecular mass of less than 400 MW. For example, Dimethyl disulfide ($(CH_3)_2S_2$, MW 94), an olfactory stimulatory compound released in the vaginal discharge of female hamsters, may play a role in attracting conspecific males (Singer et al., 1976; Petrulis and Johnston, 1994). The surface of the olfactory epithelium is covered by a thin film (about 5-10 micrometers) of watery mucus. Any molecule that stimulates olfactory receptor cells therefore must also be soluble in the mucus covering the olfactory epithelium. Compounds solubilized in this mucus membrane bind weakly to specific protein receptors, such as G-protein-linked receptors, which are embedded in the phospholipid bilayer of olfactory receptor cells (Buck & Axel, 1991). The olfactory sensory cell is a bipolar neuron, with a single axon going to the central nervous system, and a dendrite that terminates on a body surface. Once a compound is bound to a protein receptor of an olfactory cell, a message may be sent to the central nervous system through this bipolar neuron. This message is sent to the central nervous system through a signal transduction pathway. Apparently, signal transduction occurs largely in the olfactory cilia projecting from the apical (dendritic) end of the cell into the mat of mucus on the surface of the nasal lining (Gold & Nakamura 1987; Lidow et al., 1987; Kurahashi & Kaneko, 1991). Signal transduction in olfactory cells is thought to mimic that in other systems such as neurotransmitter or hormone

action and visual reception where cyclic nucleotides act as second messengers (Pace et al., 1985; Lancet, 1986; Pace & Lancet, 1986; Lancet & Pace, 1987; Anholt, 1987,1989;). In a signal transduction pathway, binding of the olfactory stimulus activates the receptor which activates a guanine nucleotide-binding protein (G-protein) (Figure 1). The activated G-protein then stimulates the increase in adenylate cyclase activity. This results in the breakdown of adenosine triphosphate (ATP) into cAMP, which directly (or indirectly) alters the conductance channels in the membrane. This may in turn cause the cell to fire. The system is recycled as cytoplasmic phosphodiesterase transforms cAMP into AMP.

The mammalian olfactory system is probably the most important system for the detection of chemosignals. This is due, in part, to the longer distance, relative to taste and the vomeronasal organ (discussed below), in which compounds may be detected. This long-distance detection may be important for location of a chemosignal source. Once the source is located, possible nonvolatile chemosignals may subsequently be detected through closer-range modalities such as taste and/or the vomeronasal organ.

In sensory physiology, taste is strictly defined as the sensation elicited by water-soluble chemical stimuli acting on the taste buds. Taste organs in most mammals are located in or near the oral cavity (especially the tongue and soft palate), pharynx, and larynx. Taste sensory cells are modified epithelial cells intervated by sensory nerves. Four distinct basic taste sensations can be distinguished, namely, sweet, sour, salt, and bitter. Sweet and bitter stimuli apparently bind to specific receptors in the taste buds that are linked to a tranducing system, such as in olfactory cells. In contrast, salt and sour stimuli

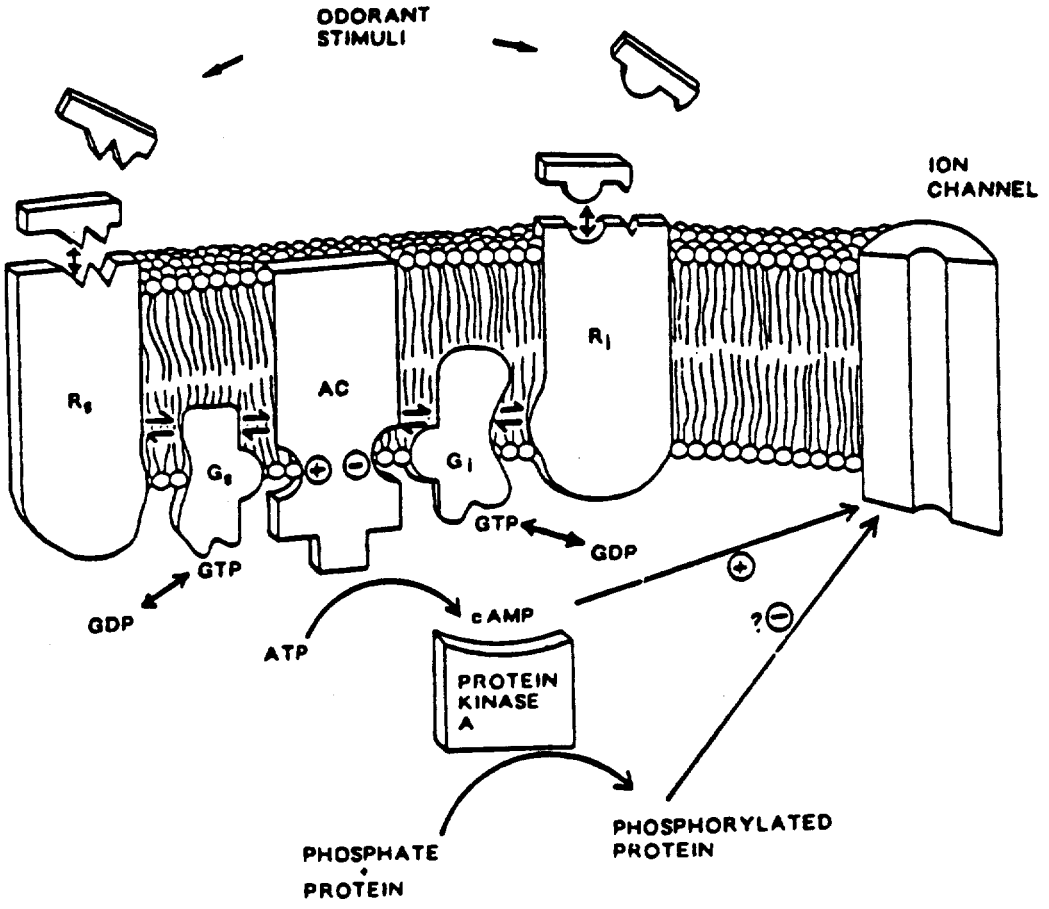

Figure 1. Model of the adenylate cyclase system. Odorant stimuli bind to protein receptor sites (Rs and Ri) within the membrane. The receptors, activated by binding to stimuli, activate G-proteins (Gs and Gi) to exchange GDP for GTP. The activated G-proteins either stimulate (+) or inhibit (-) the activity of adenylate cyclase (AC) to produce cyclic AMP (cAMP) from adenosine triphosphate (ATP). The cAMP can open (+) the ion channel directly, and/or perhaps can work indirectly through a protein phosphorylation step to close it (-) after it fires (Farbman, 1992).

do not bind to specific receptors in the taste buds and are apparently linked to membrane channels. Thus, these stimuli apparently do not work through a second messenger, transduction pathway. When stimuli reach the taste buds, a taste impulse is generated through a synapse to a sensory nerve which may then carry the information to the brain (Farbman, 1992). Taste may be important for the reception of nonvolatile, water soluble chemosignals. In addition, taste may be associated with the reception of chemosignals through a distinct chemosensory modality, located in the palate, called the vomeronasal organ.

The vomeronasal organ (historically termed Jacobson's organ) is present in most terrestrial vertebrates. The structural details of this organ vary from species to species. Vomeronasal organs (VNO) are paired, tube-shaped structures, each enclosed in a bony capsule, located in the anterior, ventral end of the nasal septum. In mammals, the VNO opens by a narrow duct either into the base of the anterior nasal cavity, e.g., rats (Farbman, 1992); or into the incisive (also called the nasopalatine) canal that connects the nasal and oral cavities, e.g., dogs (Farbman, 1992); or into the oral cavity where it is connected to the nasal cavity through accessory ducts, e.g., elephants (Rasmussen, 1994). The cells lining the vomeronasal organ (VNO) are similar to olfactory cells except that they possess, predominantly, microvilli instead of cilia (Adams and Wiekamp, 1984; Taniguchi and Mikami, 1985). The sensory cell of the VNO is a bipolar neuron, such as in olfactory cells, and axons from the VNO receptor cells form a distinct nerve tract which passes through the cribiform plate to the accessory olfactory bulb. Current research suggests that specific protein receptors exist in the cells lining the VNO (Kishimoto et al., 1994; Luo et al.,

1994). Therefore, the VNO system may possibly operate through a signal transduction pathway.

In many species, the vomeronasal organ is somewhat isolated from the airstream generated during respiration and/or sniffing. Mechanisms in which compounds, both volatile and nonvolatile, reach the sensory cells of the VNO include a "vomeronasal pump" and/or the flehmen response.

The "vomeronasal pump" is present in some mammals, e.g., hamsters (Meredith, et al., 1980). This pump operates through several thin-walled blood vessels, anterior to the VNO, under autonomic nerve control. These vessels become restricted by sympathetic intervention via stimulation of the nasopalatine nerve. This vascular constriction allows for the expansion of the VNO lumen causing a partial vacuum permitting vapors and fluids to enter it from the nasal cavity or the nasopalatine canal (Farbman, 1992). Both volatile and nonvolatile compounds are drawn into the lumen and reach the sensory cells. An alternative mechanism for the transport of chemosignals to the VNO is the flehmen response.

The flehmen response (Schneider, 1930; Muller-Schwarze, 1979; Albone, 1984) is a behavior common among mammals in which compounds are transported to the ducts leading to the vomeronasal organ. Direct contact of the VNO through the flehmen response has been demonstrated with rhodamine and sodium fluorescein (tracer dyes) in experiments with hamsters (Beauchamp et al., 1980; Wysocki et al., 1980) and male goats (Ladewig and Hart, 1980). Flehmen responses are exhibited by both male and females in response to urine and other exudates. They are characterized in many mammals by contact with the

mouth, lips, tongue, or trunk tip, with the exudate followed by a lifting or curling of the upper lip (Albone, 1984) or trunk (Rasmussen et al., 1982, 1993).

Although the VNO system may detect both volatile and nonvolatile compounds, the VNO is crucial for the detection of nonvolatile chemosignals. Nonvolatile compounds will not stimulate the olfactory system, and thus mammals must rely on the VNO system for the detection of these compounds. An example of a nonvolatile, VNO stimulating, mammalian chemosignal is a compound that elicits copulatory behavior in male hamsters (Singer et al., 1986). This compound, a protenacious aphrodisiac pheromone, is water soluble and has a molecular mass of 17 KDa. It is secreted in the vaginal discharge of female hamsters.

CHEMICAL COMMUNICATION IN ASIAN ELEPHANTS

Asian elephants (*Elephas maximus*) apparently utilize compounds present in urine (Rasmussen, 1988; Rasmussen, et al., 1982, 1986, 1993), cervical mucus (Rasmussen et al., 1993), temporal gland secretion (Rasmussen, 1988), and perhaps other exudates, to communicate conspecific information. This information is transmitted through the flehmen response, and/or olfaction, and perhaps other chemosensory modalities, such as palatal pit area contact (PPACR), scrub, and check responses. The type of information transmitted may include individual identification, long-term filial-maternal recognition, musth cycles in males, and estrous cycles in females.

Presently, a biologically active fraction from female Asian elephant estrous urine and cervical mucus has been isolated and characterized (Rasmussen et al., 1993). This fraction contains a compound(s) that elicits high frequencies of flehmen responses by mature male Asian elephants. This compound(s) is utilized by the males for the detection of estrous cycles in the females. Thus, it plays a vital role in the elephant reproductive scenario.

The general approach to the isolation of the biologically active fraction of female Asian elephant estrous urine and cervical mucus included the following. First, whole, biologically active (i.e., flehmen response eliciting among males) estrous urine was extracted with dichloromethane using specially designed liquid-liquid extractors. Retention of biological activity of these crude fractions was then verified through bioassays. Second, utilizing column (flash) chromatography, crude purification of the extracts was performed. Bioassays again confirmed the retention of biological activity of

this second fractionation procedure. Refined purification of the active fraction was conducted with high performance liquid chromatography (HPLC), and again bioassays confirmed retention of biological activity. Reverse phase HPLC revealed that the biologically active fraction was, initially, composed of one compound. A compound, indolo-[2,1-b] quinazoline-6,12-dione (tryptanthrine, MW 210) was identified by mass, proton nuclear magnetic resonance, ultraviolet-visible, and infrared spectrometries (Rasmussen et al., 1993). However, exhaustive and repetitive bioassays established that pure authentic (synthetic) tryptanthrine, by itself, was not the compound that elicited flehmen responses in the males. Rather, further HPLC purification studies indicated a coeluting fraction. This fraction elicited high frequencies of flehmen responses in the males. At this stage, it is unclear if the biologically active compound(s) is one or more components. Apparently, tryptanthrine does not play a role.

Concurrent studies with male-to-female chemical signals in male Asian elephant musth temporal gland secretion (TGS) are being conducted. Musth is an annual physiological and behavioral change, usually lasting several months, that is experienced by virtually all mature male Asian elephants (Eisenberg et al., 1971; Jainudeen et al. 1972a). The function of musth is unknown. It has been proposed that musth is either an aggressive state for males to achieve a higher dominance rank (Poole and Moss, 1981), or it is part of a mating scenario (Jainudeen et al., 1972a; Poole and Moss, 1981). Behaviorally, musth is characterized by aggression, disorientation, and/or unpredictable actions (Eisenberg et al., 1971; Jainudeen et al. 1972a; Schmidt, 1978). Physiologically, musth is marked by elevated serum testosterone levels

(Jainudeen et al., 1972b; Rasmussen et al., 1984; Niemuller and Liptrap, 1991), urine dribbling, and copious secretion of an aqueous and odoriferous fluid from the temporal gland (McGaughey, 1963; Molamure, 1969; Jainudeen et al., 1972a; Niemuller and Liptrap, 1991). The temporal gland, unique to elephants, is a modified aprocrine sweat gland located in the temporal fossa (mid-cheek) region (Eisenberg et al., 1971). Although it has been proposed that it functions as a scent gland (Buss et al., 1976), the precise function of the temporal gland is unknown. Two distinct categories of behavior in female Asian elephants to temporal gland secretion (TGS) have been observed. These categories of behavior include trunk-to-mouth actions, including the flehmen and palatal pit area contact response (Rasmussen, 1988), and avoiding reactions (Molamure, 1969; Haight, personal communication) which are apparently olfactory mediated. However, the function of these female behaviors to TGS is unknown.

BACKGROUND OF THIS RESEARCH

Based on the observation that male Asian elephant temporal gland secretion elicits specific behavior, both trunk-to-mouth behavior (Rasmussen, 1988) and avoiding reactions (Molamure, 1969; Haight, personal communication), in conspecific females, it was hypothesized that male-to-female chemical signals exist in TGS. Further, it was hypothesized that these possible chemical signals are related to musth since TGS is exuded by males only during their musth periods. Based on this hypothesis, the goal was to characterize and/or identify compounds in TGS that elicit both trunk-to-mouth responses and avoiding reactions in females.

The first study was focused on the identification of compounds present in TGS that elicited trunk-to mouth responses in females. Due to the small quantities of TGS collected each day (5-7 ml), extraction and bioassay, such as the method used for studies with female Asian elephant estrous urine, of whole TGS was not practical. In lieu of fractionation of TGS, the compounds were bioassayed, in synthetic form, that had previously been identified in male Asian elephant TGS (Rasmussen et al., 1990). These compounds were identified by gas chromatography-mass spectrometry analysis of dichloromethane extracts of whole temporal gland secretion. A total of 30 synthetic compounds, identified in TGS, were bioassayed among a group of four female Asian elephants utilizing a standard and reliable bioassay (Rasmussen et al., 1982; Rasmussen, 1988). During these preliminary bioassays, only one compound, cyclohexanone, evoked high frequencies (i.e., responses per hour) of trunk-to-mouth, both flehmen and PPACR responses by the females during repeated

bioassays. In addition, cyclohexanone elicited high frequencies of scrub and check responses. The function of PPACR, scrub, and check responses is unknown. However, they possibly have a role in inter-sexual communication based on observations of these responses to TGS and urine (Rasmussen, 1988). Based on preliminary results, it was necessary to further investigate the possible biological activity of cyclohexanone. In this study, flehmen, PPACR, scrub, and check response of four female Asian elephants were quantified to cyclohexanone.

The second study involved the isolation and identification of compounds in male musth TGS that elicited avoiding reactions in female Asian elephants. Avoiding reactions in female Asian elephants had not previously been rigorously defined in the literature (Molamure, 1969). Through observations, avoiding reactions to TGS were defined as an instantaneous and rapid backing away, involving both trunk and body movements, subsequent to presentation of TGS. In addition, it was observed that this response was periodically accompanied by rapid ear movement and vocalizations (trumpeting, growling, and squeaking).

Through the initial observations, it was hypothesized that avoidance compound(s) of TGS were volatile, and perhaps ephemeral, and that olfaction was the primary or possibly the sole chemosensory modality utilized for the detection of these chemosignals. These observations included: 1) avoiding reactions in females were elicited by freshly collected TGS. In contrast, TGS collected 1 hour prior to the bioassay did not elicit any responses in the females. In addition, previous studies had failed to demonstrate any avoiding reactions to frozen, then thawed TGS (Rasmussen, 1988); 2) no trunk contact by

the females was made to fresh TGS prior to avoiding reactions indicating the use of olfaction.

Based on the hypothesis that avoidance compounds of TGS were volatile, the compounds in the headspace of TGS were of specific interest. A method was needed to collect (in the field), store, analyze (gas chromatography and gas chromatography-mass spectrometry) and bioassay the TGS headspace compounds. A method for this purpose did not exist. In collaboration with the Global Change Research Center, Oregon Graduate Institute of Science & Technology, a method for this study was developed. A sample collection apparatus, originally used to study global methane production in termites (Rasmussen & Khalil, 1983), was modified. The original collection apparatus consisted of a glass jar with an air-tight lid perforated with a septum. The sample was placed in the jar and a headspace sample was drawn through the septum into a glass syringe. The glass syringe technique was replaced with 850 ml internally electropolished stainless steel canisters. These canisters are routinely utilized to collect air samples in global sites for trace gas analyses involved in the greenhouse effect (Khalil and Rasmussen, 1994) and the ozone hole phenomenon (Khalil and Rasmussen, 1993). Collection of the TGS headspace compounds in these special canisters was expected to provide chemical stability during storage prior to gas chromatographic and gas chromatographic-mass spectrometric analyses (Rasmussen and Lovelock, 1983). In addition, subsequent to chromatographic analyses, the TGS headspace compounds could be released for bioassay to test the retention of biological activity. Utilizing this method, a headspace fraction of biologically active

(avoidance eliciting) TGS was isolated and possible bioactive compounds in this TGS fraction were identified.

CHAPTER II

CHEMOSENSORY RESPONSES OF FEMALE ASIAN ELEPHANTS TO CYCLOHEXANONE

METHODS AND MATERIALS

Animals. Four nonrelated female Asian elephants were utilized for these studies at the Metro Washington Park Zoo, Portland, Oregon. Their ages at the time of the studies were Belle, 42; Hanako, 29; Tamba, 22; and Susie, 35 years.

Bioresponse definitions. Four bioresponses, flehmen, palatal pit area contact, scrub, and check, were recorded to placed cyclohexanone samples and control compounds. The bioresponses recorded are described as follows.

During the flehmen response the proboscideal process (the dorsal trunk tip) is placed in a liquid substance (often urine) to be assessed. The trunk is then curled and raised vertically where the trunk tip is placed on paired orifices of the incisive foramina located in the roof of the mouth (Figure 2 and 3). These orifices are contiguous with paired incisive ducts and VNO ducts that are confluent with the paired lumena of the vomeronasal organ (Rasmussen et al., 1986; Rasmussen, 1988).

The palatal pit area contact response (PPACR) begins with placement of the proboscideal process in a liquid substance (often urine) to be assessed. The trunk is then raised to the side of the mouth where the trunk tip is placed on a

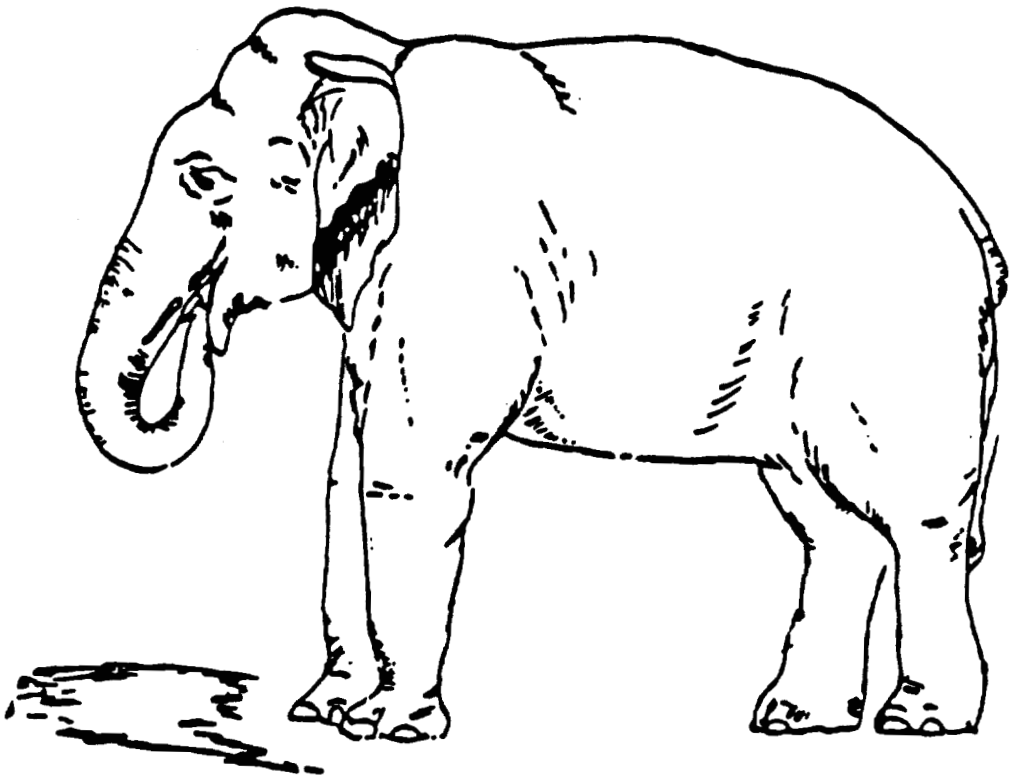

Figure 2. Female Asian elephant performing a flehmen response to liquid sample (often urine) on ground. The trunk tip with sample is touching paired orifices in palate which leads to vomeronasal organ.

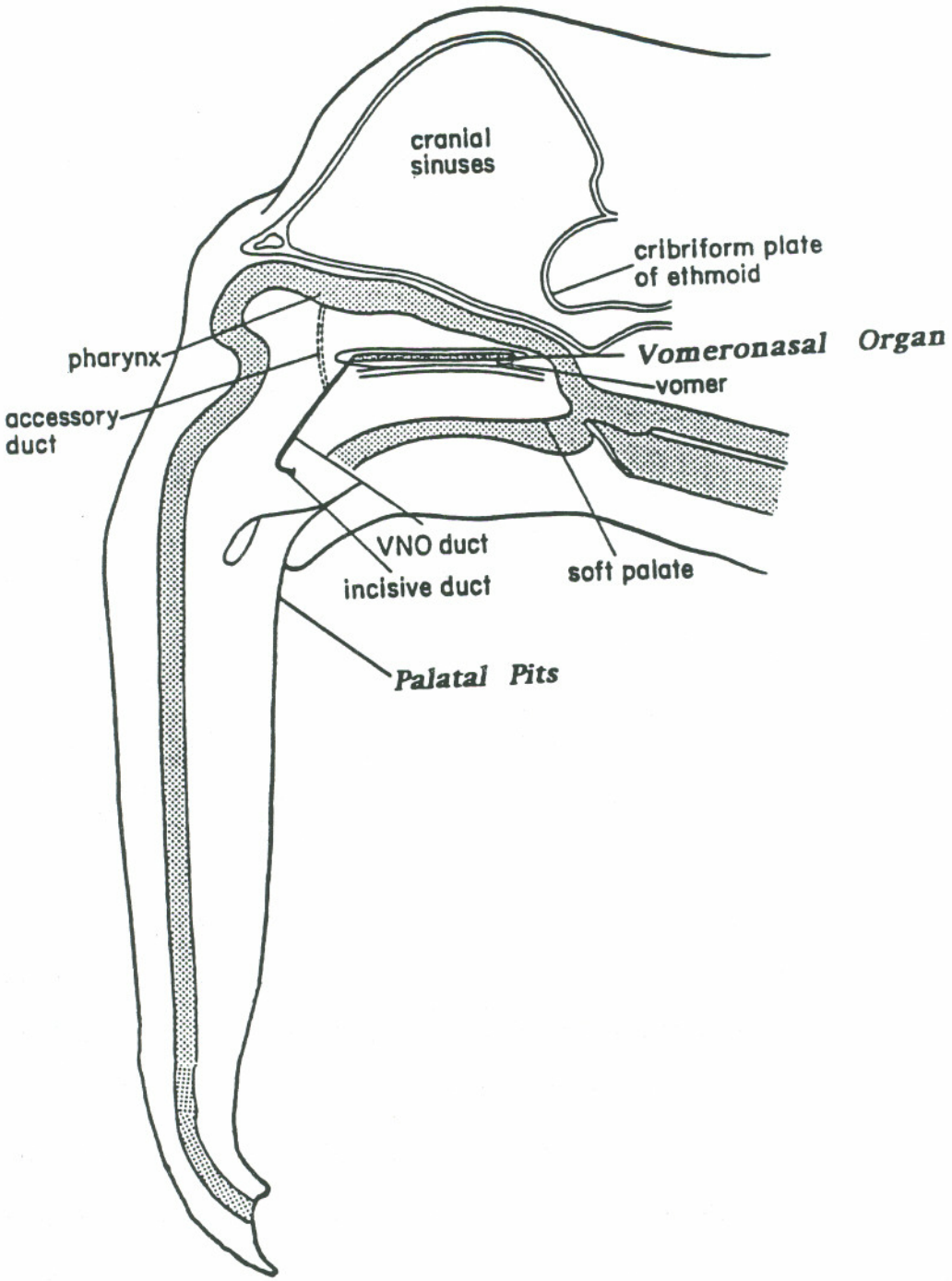

Figure 3. Paramedial view of the head of a female Asian elephant showing location of the palatal pits and the openings of the ducts leading to the vomeronasal organ.

series of small pits (Rasmussen et al., 1986; Rasmussen, 1988). These pits are located laterally at the junction of trunk and hard palate. Morphologically these pits are crypt-like structures that are lined with stratified squamous epithelium containing many lymphocytes (Haight and Rasmussen, unpublished). Among the captive Asian elephants at the Metro Washington Park Zoo, PPACR is observed often; the function of this response is under assessment. Although PPACR can be of several types, self-initiated, sequentially multiconspecific, or in response to natural or placed samples, only the latter category was recorded for this study. The location of the sensory palatal pits, is shown in Figure 3.

Scrub responses are characterized by an initial placement of the proboscideal process into a sample, either natural or placed, and an immediate flattening of the entire end of the trunk, especially the ventral region, on the sample. This is followed by a vigorous scrubbing motion using the whole trunk end. This response, often lasting several seconds, is usually longer in duration relative to the other responses (Rasmussen et al., 1986).

Check responses are defined as the placement of the proboscideal process into a sample, either natural or placed (Rasmussen et al., 1986; Rasmussen, 1988). By definition, check responses precede flehmen, PPACR, and scrub responses. Some check responses are independent, sole occurrences. During these independent check responses the proboscideal process is placed into a sample and removed but no further trunk movements occur. For this study, only independently occurring check responses were quantified. Whether check and scrub responses are olfactory, chemosensory, tactile, or a combination of these is a facet of long-term investigations.

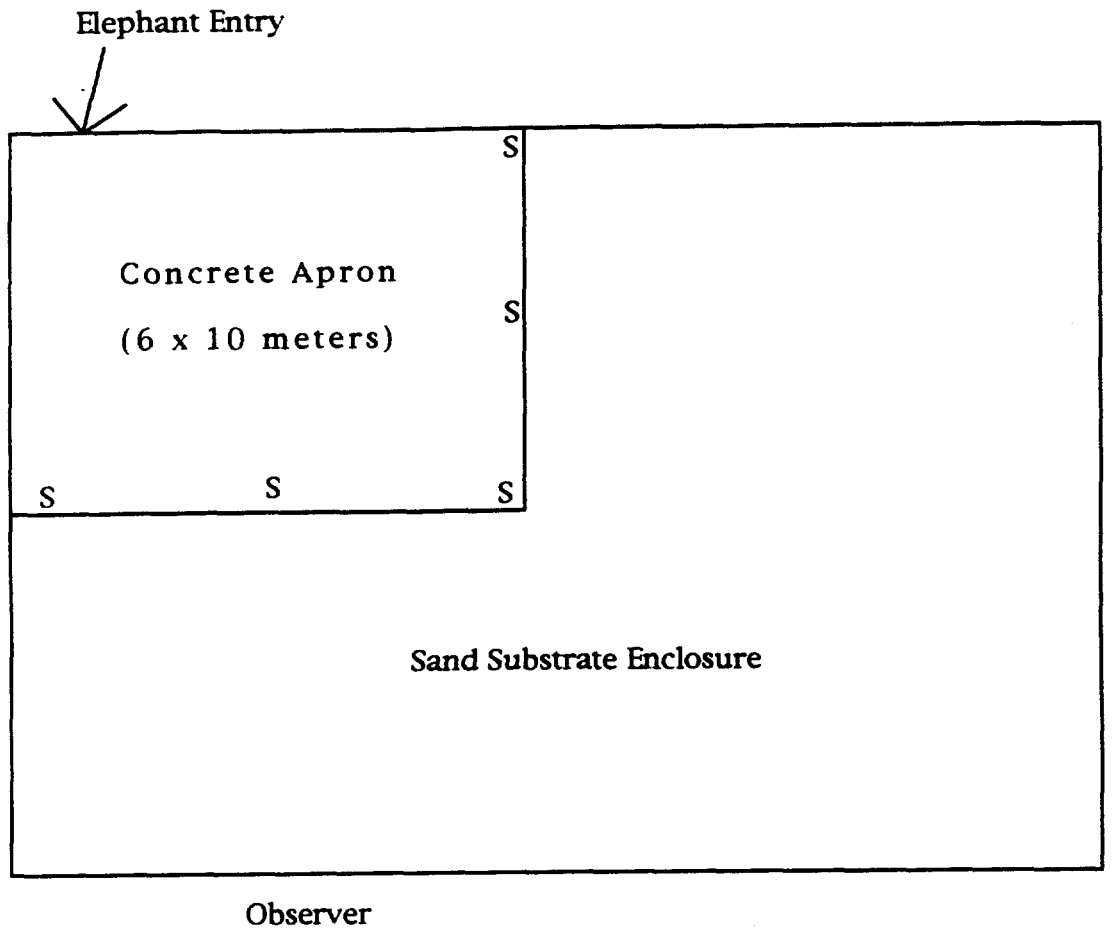

Figure 4. Diagram of 0.3-ha sand yard bioassay area. Female elephants were released from barn with immediate access to concrete apron. S indicates the sample placement positions.

Presentation of test substances. All bioassays were conducted from 9:30 - 10:30 AM in a 0.3-ha sand yard (Figure 4). At one end of the sand yard was a concrete slab (6 x 10 meters) with a roof supported by five concrete posts. The samples for bioassay were placed on this slab after it had been hosed off and allowed to dry. Samples were poured adjacent to upright posts in separate positions. Placement of the samples next to the posts gave us the clearest view of the samples. For each bioassay we used two to three different concentrations of cyclohexanone. The presentation positions of the samples, the various concentrations of cyclohexanone and the controls, were changed and noted so that the positions of each sample were different for each bioassay. The cyclohexanone and control samples were bioassayed simultaneously.

After the samples were placed, the elephants were released, as a group, from the barn to the sand yard as part of their daily routine. During the 60 minute bioassay period, an observer standing outside of the sand yard recorded all bioresponses performed by each elephant to the samples (a sample bioassay sheet is shown in Appendix A). A bioassay was considered complete only if each elephant voluntarily walked across the concrete slab at least five times to ensure ample exposure to the samples. Ten group bioassays were conducted within two months. Because of management procedures, Hanako was present only for six bioassays and Susie was present for nine bioassays.

Possible low threshold concentrations for cyclohexanone with one female Asian elephant (Tamba) was investigated. Several months after group bioassays, the cow grouping had been changed. The experimental conditions were dictated by these alterations. Only a solitary animal (Tamba) was available, at our requested time and location, for experimentation. Five

bioassays were performed, within two weeks, with this solitary animal. It was suspected that low concentrations should elicit bioresponses since there are large fluctuations in concentration of the chemical components, including cyclohexanone, of male temporal gland secretion (Rasmussen et al., 1990).

Test samples. During the 10 group experiments, cyclohexanone (analytical grade, 0.013% residue after evaporation, 0.020% water, purchased from Mallinckrodt) was bioassayed at estimated physiological concentrations (10, 20, 40, and 80 mM) based on previous analytical data. Each dilution of cyclohexanone was bioassayed three to 10 times. During the five bioassays with the solitary animal (Tamba) cyclohexanone concentrations of 0.1 mM and 1.0 mM were presented. Both concentrations were bioassayed five times.

Acetone has been used effectively as a solvent for several types of bioassays (Rasmussen et al., 1982, 1986). All samples for bioassay were dissolved in 10 ml of acetone prior to addition to 50 ml of acetate buffer, pH 6.0. The control samples for all bioassays included acetone (65 mM) and acetate buffer, pH 6.0 (200 mM).

Analysis of Data. A possible concentration effect of cyclohexanone on bioresponse frequency was analyzed. Linear regression was used to analyze the total number of bioresponses per bioassay to each concentration of cyclohexanone for each bioresponse type. This analysis was employed for group bioassays only.

Bioresponses to cyclohexanone versus controls, during group bioassays, for each elephant were also statistically analyzed. Bioresponses to cyclohexanone occurred rarely, which resulted in high (50-100%) standard errors and nonnormal data. Standard errors are not reported due to lack of meaning.

Because of nonnormal data, a one-tailed nonparametric sign test at a probability of 5% was utilized (Snedecor and Cochran, 1989). The *N* value for this test was defined as the number of bioassays with the occurrence of at least one bioresponse to cyclohexanone (not including ties with the controls). An *N* value of at least five was required to utilize this test.

Gas chromatographic analysis of cyclohexanone. The purity of cyclohexanone, used in this experiment, was confirmed by gas chromatographic (GC) analysis. A 1 mM cyclohexanone GC standard (cyclohexanone dissolved in dichloromethane) was prepared and analyzed. In addition, the possibility of cyclohexanone-derived side-product formation during bioassays, e.g., the reduction of cyclohexanone to cyclohexanol, was examined. For this experiment, a 40 mM cyclohexanone solution (cyclohexanone dissolved in 10 ml of acetone in 50 ml acetate buffer) was prepared and set outside in indirect sunlight for one hour (this step was performed to simulate sample placement on the covered concrete slab utilized for bioassay). A 50-ml aliquot of the 40 mM cyclohexanone solution was immediately placed in a separatory funnel and extracted three times with 12.5 ml dichloromethane. The three 12.5 ml dichloromethane extracts were pooled, concentrated under nitrogen, and analyzed.

The gas chromatograph was a Hewlett Packard 5790A with a flame ionization detector (FID). The GC-FID used a DB-1, 0.32-mm ID x 60-m x 1.0 micron film thickness, polymethyl silicone coated capillary column (J&W Scientific, Inc.). The FID output was processed via a Hewlett Packard 3392A integrator. On-column injection was used. The oven was temperature programmed from 32^0C to 200^0C at 4^0C per minute.

RESULTS

Gas chromatographic analysis of cyclohexanone. The GC-FID chromatogram of the cyclohexanone standard showed only two peaks, a solvent (dichloromethane) peak and a cyclohexanone peak, thus demonstrating the purity of cyclohexanone (Figure 5). The GC-FID chromatogram of the 40 mM cyclohexanone solution, set in indirect sunlight for one hour, showed two peaks. One peak was the solvent peak and the other had a similar retention time as cyclohexanone. Using peak enhancement, this peak was verified to be cyclohexanone. The results of this experiment demonstrate that side-product development during bioassays with cyclohexanone was unlikely.

Concentration Effect. During group bioassays, the concentration of cyclohexanone appeared to have no, or only slight, effect on the frequency of all bioresponse types among the females. This is indicated by the low r^2 values (Table 1A). Even though the higher (40 mM and 80 mM) cyclohexanone concentrations were bioassayed more frequently, there was no distinct linear relationship between concentration and bioresponse frequencies.

The total numbers of bioresponses by the solitary animal (Tamba) to both the 0.10 mM and 1.00 mM cyclohexanone concentrations were similar (Table 1B). The single most important result with this experiment was the fact that an elephant did respond to cyclohexanone at these lower concentrations. During these bioassays, Tamba performed a total of six check responses, and zero flehmen, PPACR, and scrub responses to the controls. Responses to controls during group bioassays are presented in the next section.

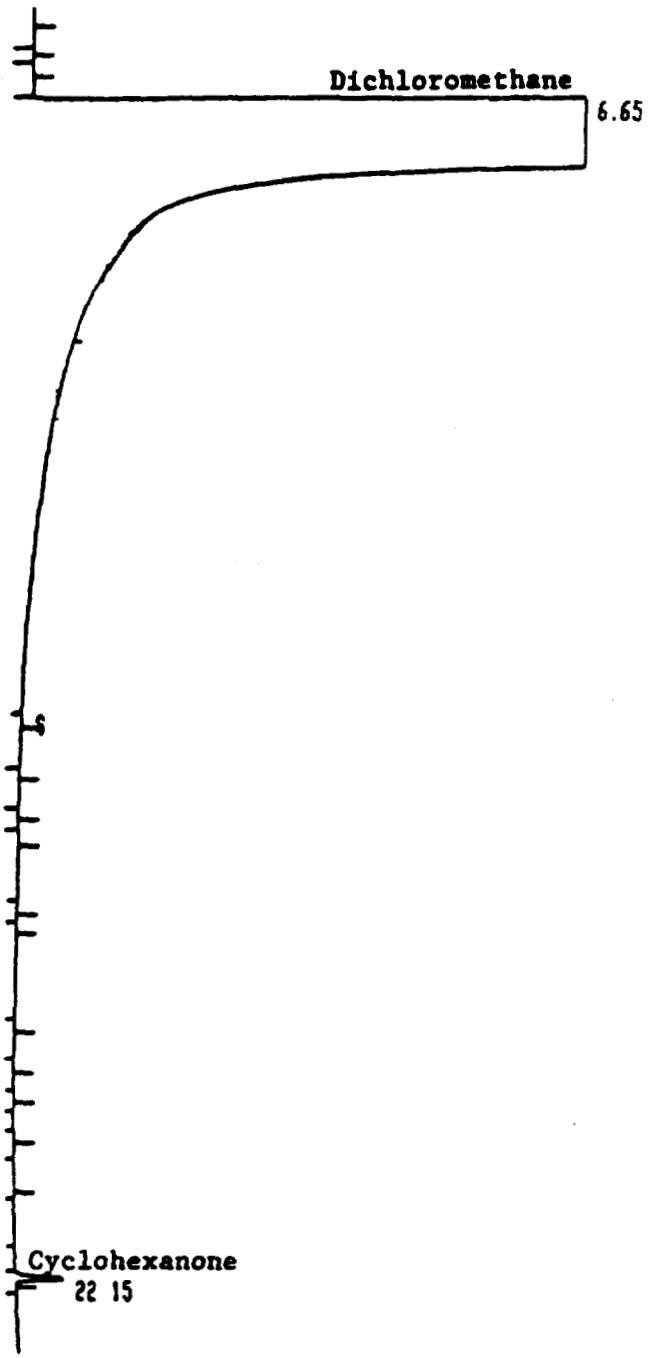

Figure 5. GC-FID chromatogram of a 1 mM cyclohexanone standard. The GC-FID was temperature programmed from 32 ^{0}C to 200 ^{0}C at 4 ^{0}C per minute. The numbers above the peaks represent retention times in minutes. One microliter of sample was loaded on the GC-FID column via an on-column injector.

Table 1. Total number of bioresponses to concentrations of cyclohexanone during 10 bioassays with a group of four female Asian elephants (A) and five bioassays with a solitary female Asian elephant (B)*

Cyclohexanone Concentration	N	Flehmen	PPACR	Scrub	Check
A. Four Elephants					
0 mM	20	0	0	0	0
10 mM	3	2	0	1	3
20 mM	5	1	4	5	4
40 mM	10	12	5	7	23
80 mM	6	8	7	7	14
R-squared		0.067	0.067	0.014	0.11
B. Solitary Elephant					
0mM	10	0	0	0	0
0.10 mM	5	2	5	5	7
1.00 mM	5	1	4	5	7

*N = Number of trials concentration was bioassayed.

Ten Bioassays with Four Female Elephants. The bioresponses to the four concentrations of cyclohexanone were pooled together for the analysis of group bioassay data because of the apparent negligible concentration effect of cyclohexanone on bioresponse frequencies (Table 1A).

Flehmen Response. The total number of flehmen responses to cyclohexanone by an individual animal ranged from zero to 14 (Figure 6). No flehmen responses occurred to the control compounds. The *N* value was high enough to statistically test one animal's (Susie) flehmen responses only. Susie performed at least one flehmen in six of nine bioassays and her flehmen frequency to cyclohexanone versus controls was significantly greater (P = 0.016, N = 6, nonparametric one-tailed sign test).

Palatal Pit Area Contact Response. The total number of PPACR by individuals to cyclohexanone ranged from zero to 11 (Figure 6). No PPACR were performed to the controls. The *N* value was high enough to statistically test PPACR of one elephant (Tamba) only. This animal exhibited at least one PPACR in seven of ten experiments. Relative to controls, the PPACR frequency to cyclohexanone by this animal was significant (P = 0.008, N = 7).

Scrub Response. On an individual basis, scrub response totals to cyclohexanone ranged from zero to 13 (Figure 6). Zero scrub responses occurred to the controls. Only Susie's scrub response frequencies to cyclohexanone were statistically testable. This animal performed at least one scrub response in six of nine bioassays. Relative to controls, Susie's scrub frequency to cyclohexanone was significantly greater (P = 0.016, N = 6).

Check Response. Check responses were the most commonly occurring response measured and occurred to both cyclohexanone and the controls

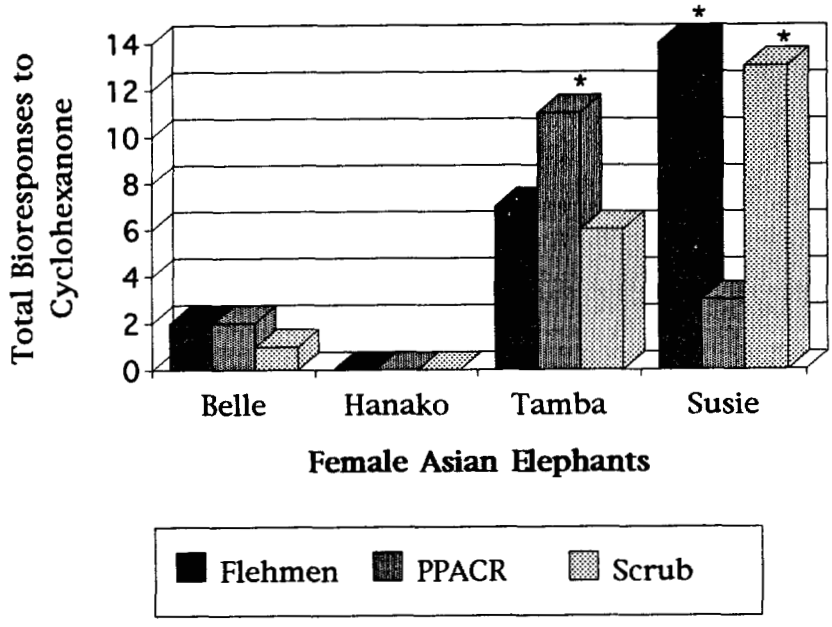

Figure 6. Total number of flehmen, palatal pit area contact, and scrub responses for each elephant to cyclohexanone during 10 group bioassays. None of these responses occurred to the control compounds. Because of management procedures, Hanako was present for only 6 bioassays and Susie was present for 9 bioassays. Susie's flehmen and scrub response totals to cyclohexanone versus controls were statistically testable, using a nonparametric sign test, and were significant (*) ($P < 0.05$). Tamba's PPACR to cyclohexanone was statistically testable and was significant (*) ($P < 0.05$) relative to controls.

(Figure 7). During all bioassays, check response frequencies by each elephant to cyclohexanone were higher than those to the controls. The check response frequencies to cyclohexanone by two elephants (Tamba and Susie) were statistically testable. Relative to controls, both Tamba's and Susie's check response frequency to cyclohexanone were significant (Tamba, $P = 0.020$, $N = 9$, Susie, $P = 0.008$, $N = 7$). Notably, Hanako performed one and only one bioresponse during all bioassays. This was a check response to cyclohexanone during bioassay number 8. During bioassays, check responses to cyclohexanone occurred in various sequences with flehmen, PPACR, and scrub responses (Appendix B).

Temporal Distributions of Flehmen, PPACR, Scrub, and Check Responses. The unique, and perhaps most important, aspect of flehmen frequencies to cyclohexanone was the temporal distribution of responses (Figure 8). The temporal distribution of flehmen responses to cyclohexanone indicated that the flehmen responses to cyclohexanone were not novel substance responses. A novel substance response is defined as a bioresponse that occurs with an elevated frequency (between three to six responses per hour) in the initial bioassay and that decreases to zero upon subsequent bioassays (usually by the third bioassay) (Rasmussen et al., 1986). Two elephant's (Tamba and Susie) flehmen responses to cyclohexanone were widely distributed. Susie was not present during bioassay 6 and this may have effected her flehmen frequencies during bioassay 7-10. Belle's flehmen response total to cyclohexanone was lowest relative to Tamba and Susie. The two flehmen responses that were performed by Belle occurred during the earlier tests.

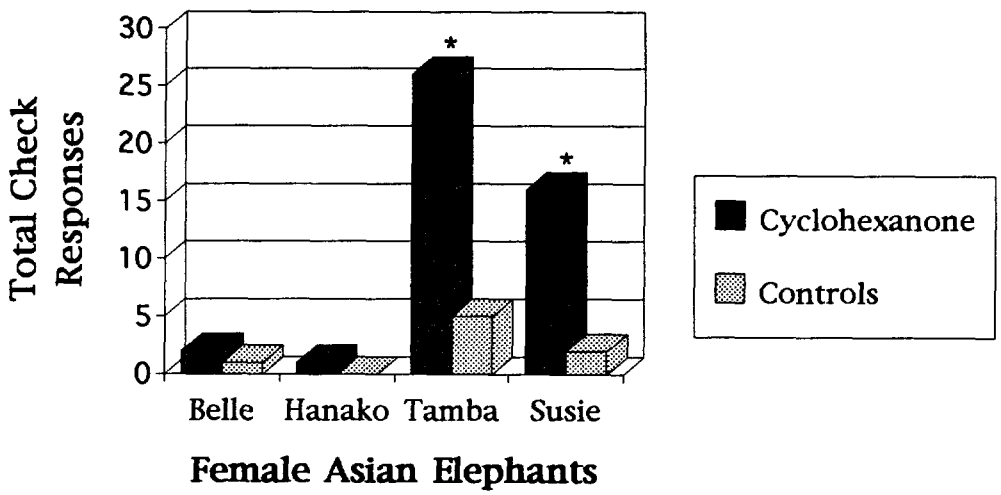

Figure 7. Total number of check responses for each elephant to cyclohexanone and control compounds during 10 group bioassays. Hanako was present for 6 bioassays and Susie was present for 9 bioassays. Tamba's and Susie's check response totals to cyclohexanone were statistically testable, using a nonparametric sign test, and were significant (*) ($P < 0.05$) relative to controls.

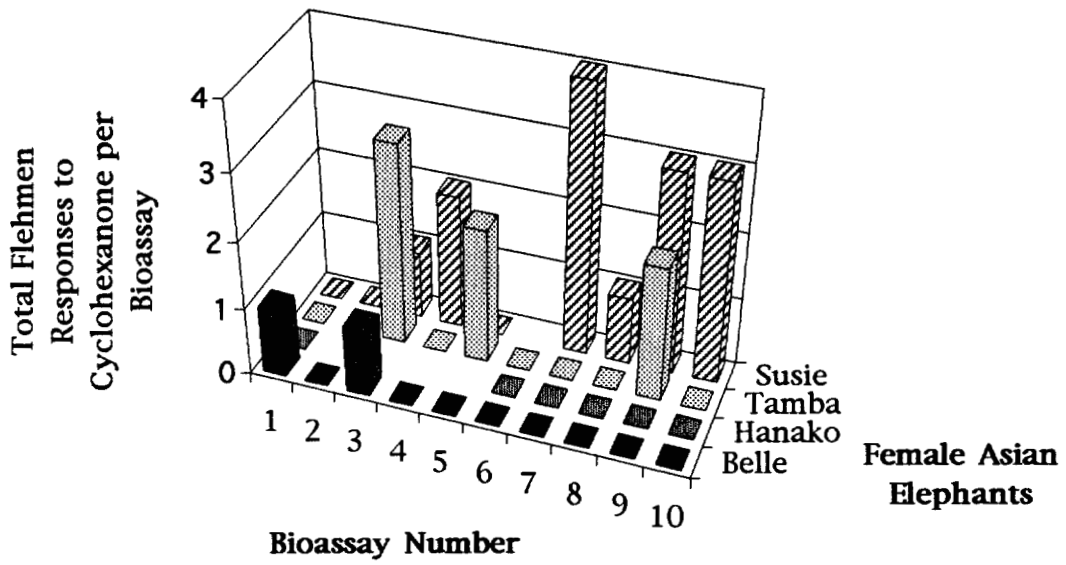

Figure 8. Plot of temporal distributions of flehmen responses performed per bioassay to cyclohexanone by each elephant during 10 group bioassays. Hanako was present for 6 bioassays and Susie was present for 9 bioassays.

This was the inverse of the flehmen response pattern demonstrated by the other two elephants (Tamba and Susie). The distributions of PPACR, scrub and check responses to cyclohexanone by the highly responsive animals (Tamba and Susie) were similar to their distributions of flehmen responses to cyclohexanone.

DISCUSSION

Cyclohexanone is the first single, synthetic compound demonstrated to elicit persistent bioresponses among elephants. In this thesis, persistent occurrences of four distinct bioresponses to this naturally-derived ketone, among a group of four female Asian elephants, was demonstrated. The uniqueness of this finding is substantiated from results of previous bioassays with 30 other single, synthetic compounds that had been identified in male temporal gland secretion (Appendix C). During these bioassays, the females either did not respond or responded with a novel substance response.

The flehmen response is a common chemosensory modality among mammals (Albone, 1984). Persistent flehmen responses to cyclohexanone, a constituent of temporal gland secretion, indicate that cyclohexanone may function as a chemical cue to female elephants. The persistence of the other bioresponses, PPACR, scrub, and check responses, to cyclohexanone also indicates some type of biological meaning for cyclohexanone since these responses occurred simultaneously with flehmen responses. It is possible that flehmen, palatal pit area contact, scrub, and check responses may be interrelated during the processing of similar chemical information (flehmen, PPACR, scrub, and check responses occurred in various sequences as reported in Appendix B). Conversely, these responses may be separate responses, occurring at the same time, processing distinct chemical information. The exact biological message that cyclohexanone may convey to females is somewhat difficult to interpret from our small group of female elephants

tested. However, it was apparent, even among our small group of elephants, that there was individual variation in response to cyclohexanone.

On examination of the individuality of bioresponses to cyclohexanone, it was hypothesized that dominance may have influenced the bioresponse patterns to cyclohexanone among this particular group of female elephants. Because of the small sample size, this possible correlation was extremely difficult to test statistically. However, this hypothesis is supported by recent studies with cyclohexanone among 21 female Asian elephants at several different facilities (Appendix D). Subordinate females apparently responded to cyclohexanone with greater frequencies relative to dominant elephants. The greatest frequencies of bioresponses to cyclohexanone among our group occurred among Tamba and Susie who were subordinate elephants relative to Belle and Hanako. The flehmen response pattern to cyclohexanone of the matriarch, Belle, was inversely related to the flehmen response pattern of the subordinate animals. In addition, the second most dominant elephant (Hanako) did not exhibit any flehmen, PPACR, or scrub responses to cyclohexanone, but performed one check response during the eighth bioassay. The low level of response by Hanako could possibly be explained by her dominance, or her individualistic, generally nonresponsive trunk-to-mouth behavior to both naturally occurring and placed samples (Rasmussen and Haight, personal communication). Currently, experiments are being undertaken to investigate response patterns by females to placed samples (urine and synthetic compounds) in relation to dominance and estrous cycles. In addition to dominance, it is possible that the stages of estrous may also be related to female response patterns to natural exudates and synthetic compounds.

Although there was individual variation in response to cyclohexanone, the concentrations of cyclohexanone apparently had no or only slight effect on bioresponse frequencies during group bioassays. It was hypothesized that bioresponse frequencies by the females would increase with increasing concentrations of cyclohexanone. This hypothesis was based on general principles of olfactory reception. In general, a threshold concentration of stimulus must be reached in order for an olfactory neuron to fire. Once the threshold of the stimulus is reached, olfactory stimulation increases, i.e., neurons fire more rapidly, with increasing concentration of stimulus. During group bioassays, increased bioresponse frequencies to increased concentrations of cyclohexanone were not observed. Only a narrow range of cyclohexanone (an eight-fold variation) was bioassayed among the group and therefore a strong effect of cyclohexanone concentration on bioresponse frequencies was not expected. However, persistent bioresponses by the females, during group bioassays, to cyclohexanone indicate that threshold concentrations of cyclohexanone were obtained. Important information regarding low threshold concentrations of cyclohexanone was learned from experiments with the solitary animal (Tamba). Apparently, persistent bioresponses by the solitary animal occurred to cyclohexanone concentrations that were 10 and 100-fold lower than cyclohexanone concentrations utilized during group bioassays. These results indicate that there may be a very low threshold concentration of cyclohexanone that stimulates bioresponses. The results of bioassays with the solitary animal (Tamba) provide valuable information for the experimental design of future bioassays with cyclohexanone.

Concentrations of cyclohexanone similar to those bioassayed among our group of females have been bioassayed among male Asian elephants. During these experiments, one of three adult males demonstrated a novel substance response to cyclohexanone, while the other two showed no responses during 20 tests (10 each) over a period of four years (Rasmussen, unpublished). Based on these results, it is apparent that cyclohexanone is a male-to-female chemosignal that may function to signal musth cycles in Asian bull elephants.

CHAPTER III

MALE TO FEMALE CHEMOREPELLENTS FROM MUSTH TEMPORAL GLAND SECRETION IN ASIAN ELEPHANTS

METHODS AND MATERIALS

GC-FID and GC-MS systems. The gas chromatograph (GC) used for preliminary analyses of TGS headspace canister samples was a Hewlett Packard 5790A with a flame ionization detector (FID). The column used was a DB-1, 0.32 mm ID x 60 m x 1.0 micron film thickness, polymethyl silicone coated capillary column (J&W Scientific, Inc.). The FID output was processed via a Hewlett Packard 3392A integrator. The sample introduction system employed a Carle® 6-port valve in line with a U-tube cryogenic trap (0.125-inch OD x 9-inch) containing 60/80 mesh glass beads. The GC oven was temperature programmed from -60^{0}C to 200^{0}C at 4^{0}C per minute, with a 5 minute initial hold at -60^{0}C.

More specific analyses were obtained with a gas chromatographic-mass spectrometric (GC-MS) system. This system consisted of a Hewlett Packard 5890A GC and a 5970B Hewlett Packard mass spectrometer. The GC used a DB-1, 0.25 mm ID x 60 m x 1.0 micron film thickness, polymethyl silicone coated capillary column (J&W Scientific, Inc.). The temperature program and sample introduction procedure was identical to that used in the GC system above. The mass spectrometer was programmed for a mass ion scan of 33-300. This allowed

for the identification of compounds from C_3 through C_{14} down to levels as low as 0.10 ppbv concentration. Compounds were identified using a NBS 75 K Hewlett Packard MS Chem Station library search, and were manually re-checked with NIST/EPA/NIH Mass Spectral Data Base Version 4.01.

Sample collection apparatus and operation procedure. The headspace sampling apparatus (Figure 9) contained two 850 ml stainless steel (type 304) canisters and a 400 ml glass jar with an air-tight lid perforated with two Swagelok® bulkhead O-ring fittings for a gas tight seal. The canisters were 100 % internally electropolished by the Summa® process (Winberry et al., 1990a) and were fitted with ultra-clean Nupro® SS-4H4 bellow-stem valves. Electropolishing passivates the internal stainless steel surface by forming an enriched chrome oxide layer so that compounds do not readily adhere or react to this surface. This improves the recovery of compounds for subsequent chromatographic analyses and bioassay. The purge canister was filled to 30 psig with zero air (manufactured air with very high purity) and was connected to the jar at one of the Swagelok® fittings, the flushing port, via a 12-inch x 0.25-inch OD piece of Teflon® tubing. From this fitting, a glass tube, 0.25-inch OD, extended to within an inch from the bottom of the jar. Although compounds from the ambient air will inevitably show up in the chromatographic analyses due to the sensitivity of the analytical procedure, purging the system with zero air reduces the concentration of these background compounds to insignificant levels. The receiving canister was evacuated to -30-inches Hg vacuum for sample collection and storage and was connected to the other fitting on the jar. This fitting, which is the headspace sampling port, was flush with the inside of the lid, i.e., it did not extend into

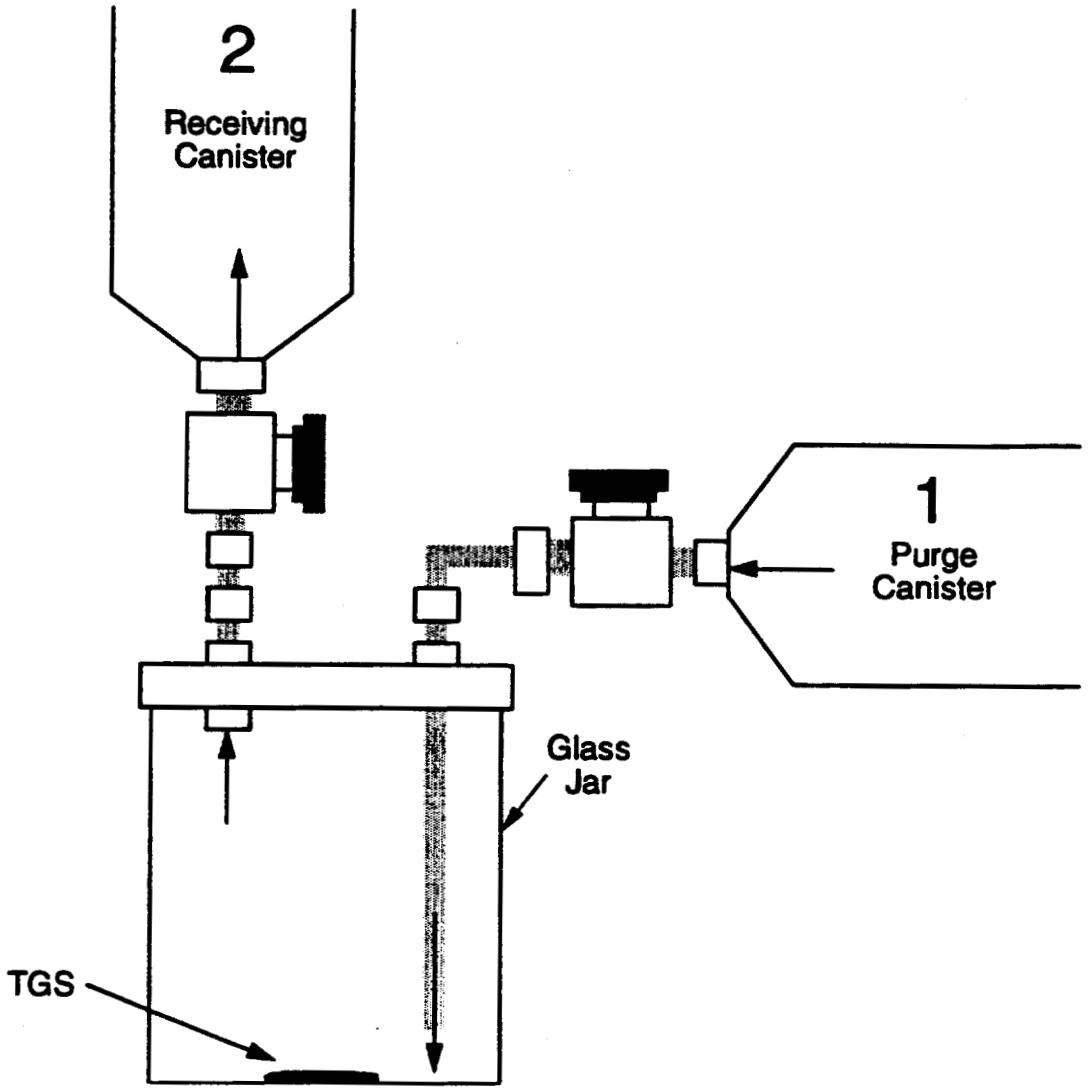

Figure 9. Diagram of sample collection apparatus showing the positions of the purge canister (canister 1) and the receiving (sample) canister (canister 2). The arrows indicate the directions of air flow.

the jar, so that the headspace sample was drawn from the top of the jar (Figure 9).

The headspace sampling procedure is described as follows. With the lid removed, 1 ml of fresh TGS, taken directly from the elephant, was placed in the jar. The lid was immediately replaced and fastened. The purge canister was securely fastened to the flushing port, and the receiving canister was connected loosely (finger tight) to the headspace sampling port. The valve of the purge canister was opened allowing zero air to flow down the glass tube to the bottom of the jar, over the TGS material, and returning to exit at the headspace port. This resulted in a flush to remove any residual air contaminants. The valve of the purge canister was then closed, the connection between the receiving canister and the headspace port was securely tightened, and the TGS compounds were allowed to equilibrate with the headspace. At 10 min., a TGS headspace sample was drawn into the evacuated canister when the valve of this canister was opened. Upon removal from the jar, the receiving canisters were pressurized with helium to 30 psig to facilitate chromatographic analyses and improve long-term storage. The jar, lid, and fittings of the apparatus were washed, rinsed with deionized water, and baked in a high temperature oven before re-use. The Teflon® tubing of the apparatus was purged with zero air for five minutes, at an elevated temperature (100 ^{0}C), before re-use.

GC-FID analysis of polar compounds in dry and moist canisters. Although electropolished and passivated, the internal walls of the stainless steel canisters still have some degree of wall adsorption. Compounds, especially polar compounds, will bind to this surface leading to poor recovery

of the sample compounds from the canisters during the chromatographic procedures and bioassays. A moist internal environment will greatly improve the recovery of the compounds withdrawn from the canisters. The hypothesized mechanism is that moisture will preferentially bind to the reactive sites on the stainless steel surface permitting the polar compounds to be much less adsorbed on the internal walls. The presence of moisture in the headspace over the TGS samples would be from the TGS material itself because it is mostly water. However, an experiment was performed to demonstrate the necessity of a moist internal canister environment for recovery of polar compounds from a National Institute of Standards and Technology calibration standard.

Six canisters, initially under high vacuum, were utilized for this experiment. Three canisters were injected with 0.5 ml of deionized water and the other three were not. All six cans were subsequently filled with a polar compound standard, prepared in dry nitrogen, in the low ppbv range containing: acetone, 2-propanol, 2-butanone, and 1-butanol[1]. Each canister was pressurized with helium to 60 psig and analyzed by GC-FID within a twenty-four hour period. A mean and standard deviation of the peak area response for each compound was determined from multiple analyses.

Control samples. Control blanks were run on the headspace of the TGS apparatus without any TGS sample. The results demonstrated that contamination from this possible source was minimal. One contaminant, dichloromethane, did show up in these control blanks. Dichloromethane is

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[1]This was a standard gas mixture traceable to the National Institute of Standards and Technology.

routinely utilized for organic extractions in our laboratory. The TGS apparatus is stored in our laboratory where high levels of dichloromethane in the lab air probably got into and adhered to the apparatus.

Collection of temporal gland secretion. Twenty-one samples of 5 to 7 ml volume of fresh TGS were collected from a 28 year-old male Asian elephant (Tunga) during an entire musth cycle (three months) at the Metro Washington Park Zoo, Portland, Oregon. For collection, the animal was loaded into a squeeze chute (a hydraulic operated restrainer) in the mid-morning and several TGS samples were collected per week throughout the three month study period. A beaker was placed under the temporal gland (mid-cheek region), and the gland was gently pressed several times causing secretion to flow into the beaker. Immediately after collection, 3 ml of the freshly collected TGS were set aside for bioassay, and 1 ml was placed in the sample collection apparatus for a headspace sample. TGS fluid has a high water content. The water in the TGS very quickly saturated the headspace in the sample jar of the apparatus and would ultimately adhere and further passivate the internal stainless steel walls of the sample canisters. Thus, it was not necessary to pre-inject deionized water into the sample canisters. The remainder of freshly collected TGS was frozen in liquid nitrogen for future experiments.

Bioassay of fresh temporal gland secretion. The bioassay of fresh TGS consisted of controlled air flow (zero air) passing over the TGS exiting through a 4-inch opening in the wall of a 20 x 40 ft. room containing four female Asian elephants (Belle-42; Hanako-29; Me-Tu-30; and Sunshine-9 years old). The observer had a safe and inconspicuous position with an unobstructed

view of all animals via an elevated platform. When an elephant placed her trunk on or near the hole in the wall, air flow (1 liter of zero air per minute) over the TGS material was initiated and avoiding reactions were monitored for that animal. A sample was only considered biologically active if at least one animal exhibited instantaneous retreat subsequent to exposure to the air flowing over the fresh TGS. Ear movement and vocalization responses (trumpeting, squeaking, and growling) were recorded and are reported in Appendix E. The study time (20 min. duration) was considered complete when all animals were exposed once to the TGS sample. A total of 21 bioassays with fresh TGS were conducted. Results showed that 11 of the 21 TGS samples collected were biologically active, i.e., elicited avoiding reactions.

GC-FID and GC-MS analyses of temporal gland secretion headspace canister samples. A small aliquot (200 ml) from each TGS headspace canister sample was analyzed by GC-FID within a month from the time of collection. After GC-FID analyses, these canisters were stored for one year at room temperature.

At the end of the one year storage period, the TGS headspace canister samples were analyzed by GC-MS. The behaviorally characterized canisters (21) were divided into two groups. The eleven bioactive samples were in one group, and the ten inactive samples were in the other group. The total non-methane organic carbon (TNMOC) in these samples was determined using the U.S. Environmental Protection Agency (EPA) Compendium TO12 method (Winberry et al., 1990b). This was done to determine the total concentration of the analytes so that the optimum amount of sample could be loaded on the column for GC-MS analyses. The results of the EPA TO12 analyses indicated that

there was not enough material in the canisters for both GC-MS analyses and subsequent bioassays. To increase the amount of material, the 11 bioactive canister samples were cryogenically transferred (pooled) into a single 850 ml canister, and the 10 inactive canister samples were pooled into another 850 ml canister. An EPA TO12 analysis was conducted on both of these pooled samples and it indicated that there was adequate quantity of material for both GC-MS analyses and subsequent bioassays. Four micrograms of the pooled active and the pooled inactive TGS headspace samples were each separately analyzed.

Bioassay of pooled temporal gland secretion headspace material stored for one year. The pooled bioactive and inactive TGS headspace material was bioassayed. Because of the limited amount of material, the original bioassay procedure was modified. A purge-tee assembly, with a toggle valve and 0-60 psig gauge, was connected to the canisters (Figure 10) and manually placed the canisters under the trunk orifice of the same four female elephants utilized in initial bioassays. A brief purge of sample (approximately 400 ml of air) was administered to the opening of their trunks when the toggle valve was depressed. A canister of zero air was the control. One bioassay per animal was performed due to the limited availability of sample.

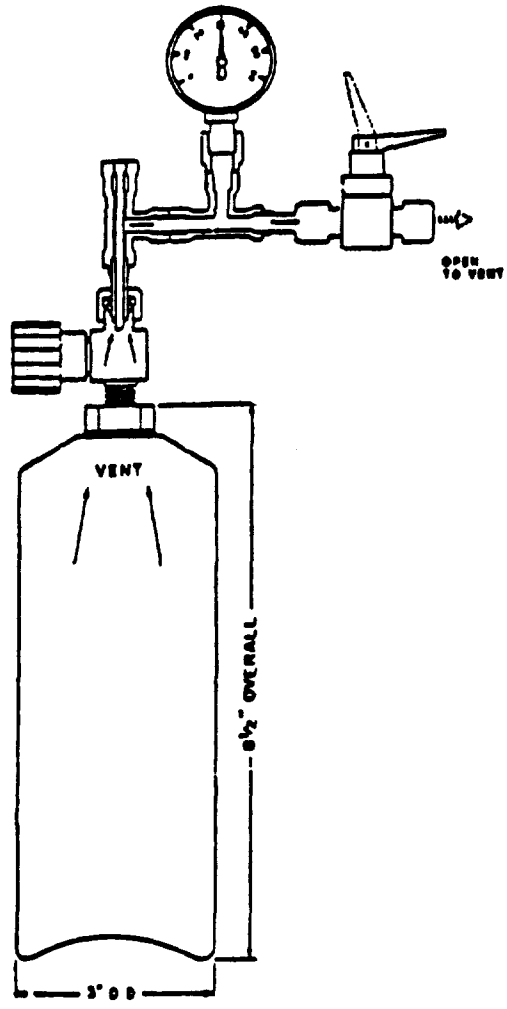

Figure 10. Purge Tee-850 ml stainless steel canister assembly. When the toggle switch of the purge tee assembly is closed and the Nupro® SS-4H4 valve of the canister is open, the pressure in the canister is registered on the 0-60 psi gauge. The contents in the canister are released when the toggle switch of the purge tee is depressed, such as during a bioassay.

RESULTS AND DISCUSSION

Atmospheric compounds identified in the headspace of TGS. The compounds identified in the TGS headspace that were in the background ambient air are listed in Table 2. These compounds are commonly occurring in the atmosphere and were generally in concentrations of less than 1 ppbv.

GC-FID analysis of polar compound standard in dry and moist canisters. Four compounds, acetone, 2-propanol, 2-butanone, and 1-butanol, were tested for recovery from moist and dry canisters. The percent recovery of these compounds in the dry canisters was considerably reduced (27% to 93%) in comparison to the percent recovery demonstrated in the moist canisters (Table 3). In addition, in the moist canisters, the standard deviations of the mean recovery of compounds were smaller relative to the standard deviations of the mean recovery of compounds in dry canisters (Table 3). The smaller standard deviations of the mean recovery from the moist canisters indicates that moisture inside the canisters increases the reproducibility of recovery of compounds during repeated GC-FID analyses. The importance of moisture inside the canisters for optimum recovery of compounds for analyses and bioassay was verified.

Bioassay of fresh temporal gland secretion. Backing up responses to fresh TGS occurred primarily among Hanako and Me-Tu (Table 4). This individuality of response patterns to TGS samples by various female elephants may be resultant from several factors. As demonstrated in Chapter II, females of subordinate rank respond to cyclohexanone, a natural component of TGS, more frequently than dominant females. The matriarch (Belle) backed away

Table 2. Common atmospheric gases identified in the background air of TGS headspace samples*

Compound	Relative Retention Time (min)	CAS #
Carbonylsulfide	10.60	463-58-1
Propene	10.80	115-07-1
2-Methylpropane	17.70	75-28-5
2-Methyl-1-propene	19.90	115-11-7
1-Butene	20.00	106-98-9
Butane	21.00	106-97-8
trans-2-Butene	21.80	624-64-6
cis-2-Butene	23.10	590-18-1
Acetonitrile	26.40	75-05-8
Trichlorofluromethane	28.00	75-69-4
Pentane	29.50	109-66-0
2-Methyl-1,3-butadiene	29.80	78-79-5
1,1-Dichloroethene	30.40	75-35-4
1,1,2-Trichloro-1,2,2-trifluroethane	31.80	76-13-1
Benzene	40.80	71-43-2
Heptane	44.00	142-82-5
Methylbenzene	47.60	108-88-3
1,4-Dimethylbenzene	53.70	106-42-3
1,2-Dimethylbenzene	55.10	95-47-6
Nonane	55.70	111-84-2
1,2,4-Trimethylbenzene	60.30	95-63-6
Decane	60.80	124-18-5
Undecane	65.40	1120-21-4

*Compounds were generally in concentrations of less than 1 ppbv.

Table 3. GC-FID analysis of polar compound standard in moist and dry 850 ml internally electropolished stainless steel canisters*

Compounds	Concentration (PPBV)	Peak Area Response: Moist Canisters Mean	Moist Canisters % SD	Dry Canisters Mean	Dry Canisters % SD	% Lost Relative to Moist Canisters
Acetone	47	45499	9	15055	24	67
2-Propanol	50	24262	15	17791	60	27
2-Butanone	48	81862	6	5500	33	93
1-Butanol	52	32953	11	6806	49	79

*This was a standard gas mixture traceable to the National Bureau of Standards, which is now the National Institute of Standards and Technology. Mean peak area response values were determined from analyses of three canisters of the standard in both moist and dry canisters. All samples were prepared and analyzed in <24 hour period. SD=Standard deviation.

Table 4. Summary of 21 bioassays of fresh TGS with four female Asian elephants*

A. TGS samples scored as biologically active

		Backing Up Responses										
Elephants	TGS sample #	1	2	3	4	5	6	7	8	9	10	11
Belle		-	nt	-	-	-	-	-	-	+	nt	nt
Hanako		+	+	+	+	+	+	+	+	nt	+	+
Me-tu		+	-	+	+	+	+	+	+	nt	-	+
Sunshine		-	nt	-	-	-	-	-	-	-	nt	nt

B. TGS samples scored as biologically inactive

	Backing Up Responses									
TGS sample #	1	2	3	4	5	6	7	8	9	10
Belle	-	-	nt	-	-	-	-	-	nt	-
Hanako	-	-	-	nt	-	-	nt	-	-	nt
Me-tu	-	-	-	nt	-	-	nt	-	-	nt
Sunshine	-	-	nt	-	-	-	-	-	nt	-

*Belle, 42; Hanako, 29; Me-tu, 30; and Sunshine, 9 year-old female Asian elephants, and "+" = avoiding reaction observed, "-" = no avoiding reaction observed, and nt = not tested.

from only one TGS sample. In addition, this elephant has an arthritic condition that may have affected her ability to back up. Finally, the youngest female (Sunshine) did not back away from any fresh TGS samples. Sunshine was the only animal who had not been exposed to a male elephant prior to experiments with fresh TGS. Perhaps the lack of exposure to a male elephant affected this animal's response pattern to TGS.

Bioassay of pooled temporal gland secretion headspace material stored for one year. During the bioassays, no animals responded to the inactive TGS sample. Likewise, the zero air control elicited no responses. In contrast, three of the four animals displayed an avoiding reaction to the pooled bioactive TGS headspace material (Table 5). The animal (Belle) who did not avoid the pooled bioactive TGS sample was generally non-responsive to all freshly collected TGS samples as mentioned above. This animal's unresponsiveness to the bioactive TGS sample is possibly attributed to this individual's behavior (as mentioned above). Interestingly, Sunshine, who did not back away from any of the fresh TGS samples, one year later exhibited an avoiding reaction to the presented pooled bioactive TGS headspace sample. Between presentations of fresh TGS and the pooled canister samples, this female was exposed to a bull elephant for the first time. Thus, Sunshine's response pattern to TGS may be related to experience with a bull and/or maturation. Based on the above results, it can be concluded that the TGS compounds stored at room temperature for one year retained their biological activity.

Preliminary GC-FID analysis of behaviorally characterized TGS headspace sample canisters. The GC-FID chromatograms of all bioactive

Table 5. Summary of bioassays among four female Asian elephants with pooled TGS headspace canister samples stored for one year*

Elephants	Backing Up Responses		
	Bioactive TGS	Inactive TGS	Zero Air
Belle	-	-	-
Hanako	+	-	-
Me-tu	+	-	-
Sunshine	+	-	-

*Belle, 43; Hanako, 30; Me-tu, 31; and Sunshine,10 year-old female Asian elephants, and "+" = avoiding reaction observed, and "-" = no avoiding reaction observed. Sunshine was exposed to a male elephant for the first time prior to this experiment.

TGS samples were similar. Likewise, the GC-FID chromatograms of all inactive TGS were similar. However, differences between the GC-FID chromatograms of bioactive and inactive TGS samples were evident. The number and area of the peaks were greater in the GC-FID chromatograms of bioactive TGS relative to the GC-FID chromatograms of inactive TGS samples (Figure 11).

GC-MS analysis of the pooled biologically active and inactive temporal gland secretion headspace canister samples stored for one year. The compositions of the pooled TGS samples, as determined by GC-MS, were similar to the compositions of the singular TGS samples measured at the time of collection (one year earlier) by GC-FID. Fifty-two compounds, ranging from 44 to 142 MW, were identified in the headspace of freshly collected TGS. Using GC-FID standards, it was determined that all compounds identified ranged in concentrations from 2 ppbv to 6 ppmv. Twenty of the identified compounds were present in both the pooled active and inactive TGS samples (Table 6).

Twenty-three compounds were unique to the bioactive TGS sample (Table 7). By retention time comparisons of the peaks of the GC-FID chromatograms, it was apparent that 10 of these twenty-three compounds were present in all 11 bioactive TGS samples. These 10 compounds were in a localized region, 40 to 58 minute retention time, of the GC-MS chromatogram (Figure 12) and were in an approximate concentration range of 2 ppbv to 130 ppbv. Perhaps one or more, or specific combinations, of these 10 compounds elicited the avoiding reactions in the females. Among these 10 compounds, the compound identified as frontalin (1,5-Dimethyl-6,8-dioxabicyclo [3.2.1] octane), was structurally the

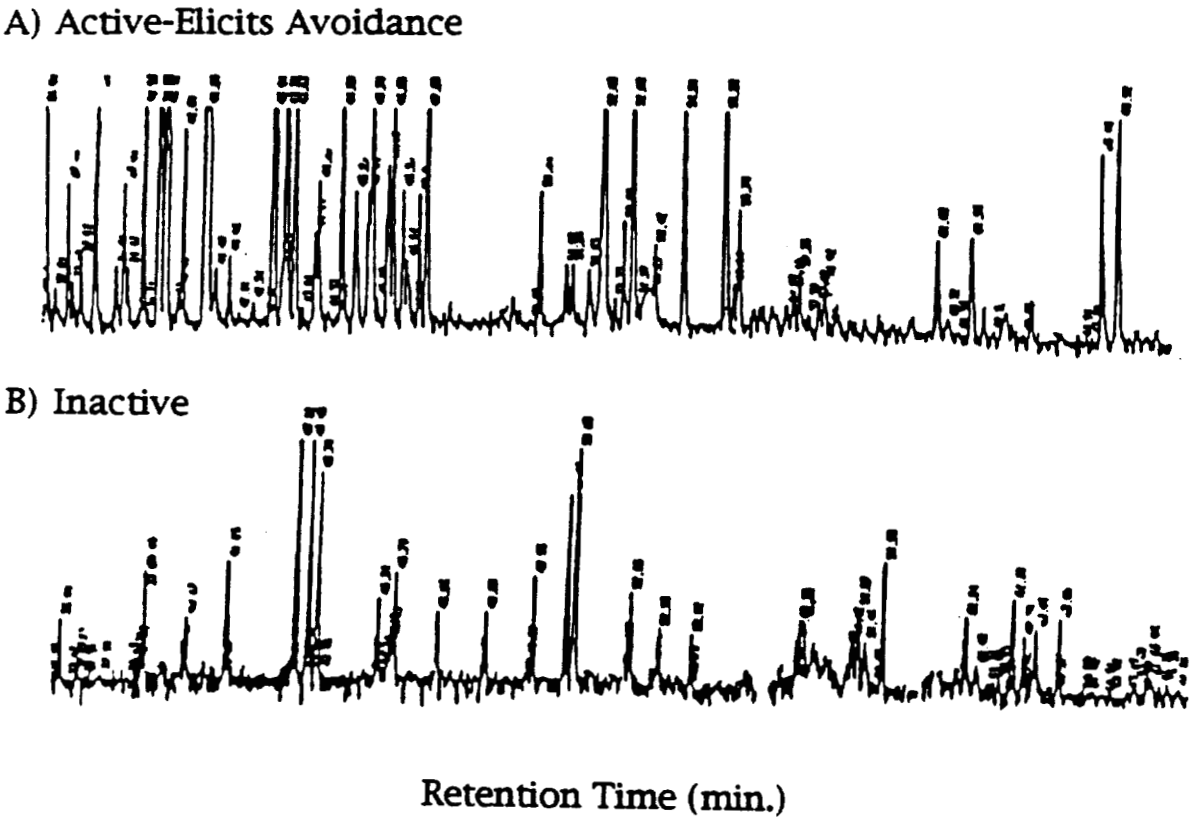

Figure 11. Representative GC-FID chromatograms of the headspace of bioactive (A) and inactive (B) TGS. The GC was temperature programmed from -60 ^{0}C to 200 ^{0}C at 4 ^{0}C per minute with a 5 minute initial hold at -60 ^{0}C. Two hundred ml of sample was loaded onto the GC column for analysis.

Table 6. Twenty compounds identified by GC-MS that were present in both the pooled bioactive and inactive TGS headspace samples*

Compound	Relative Retention Time (min)	CAS #
Acetaldehyde	18.10	75-07-0
Ethanol	26.20	64-17-5
2-Propanone (Acetone)	27.50	67-64-1
2-Propanol	29.40	67-63-0
Carbon disulfide	31.20	75-15-0
2-Methylpropanal	32.80	78-84-2
Methacrolein	33.60	78-85-3
Methyl vinyl ketone	34.50	78-94-4
Butanal	35.40	123-72-8
2-Butanone	35.70	78-93-3
2-Butanol	36.70	78-92-2
3-Methylbutanal	40.00	590-86-3
3-Methyl-2-butanol	42.30	598-75-4
2-Pentanol	43.70	6032-29-7
Dimethyl disulfide	45.80	624-92-5
2-Heptanone	54.00	110-43-0
6-Methyl-2-heptanone	57.60	928-68-7
Benzaldehyde	57.90	100-52-7
Acetophenone	63.30	98-86-2
2-Nonanone	64.50	821-55-6

*These compounds were apparently present in most of the TGS headspace samples via retention time comparisons of the GC-FID chromatograms.

Table 7. Compounds identified by GC-MS that were present only in the pooled bioactive TGS headspace sample

Present in all 11 bioactive TGS samples*

Compound	Relative Retention Time (min.)	CAS #
3-Methyl-2-butanone	40.10	563-80-4
2-Pentanone	41.90	107-87-9
3-Pentanone	42.70	96-22-0
4-Methyl-2-pentanone	45.30	108-10-1
3-Methyl-2-pentanone	45.90	565-61-7
2-Methyl-3-pentanol	47.80	565-67-3
Cyclopentanone	47.80	120-92-3
3-Hexanone	48.00	589-38-8
2-Hexanone	48.30	591-78-6
1,5-Dimethyl-6,8-dioxabicyclo [3.2.1] octane (Frontalin)	57.60	28401-39-0

Present in five or less of the 11 bioactive TGS samples*

2-Methyl-2-butanol	38.00	75-85-4
2-Methyl-3-pentanone	45.80	565-69-5
4-Methyl-1-pentanol	47.10	108-11-2
4-Methyl-2-pentanol	49.80	108-11-2
4-Heptanone	53.10	123-19-3
2-Decanone	68.50	693-54-9

Present in one of the 11 bioactive TGS samples*

1,3-Butadiene	20.30	106-99-0
Propanal	27.60	123-38-6
Furan	28.70	110-00-9
2-Methyl-2-propanol	31.20	75-65-0
2-Methylfuran	37.10	534-22-5
2-Butenal	38.00	4170-30-3
2-Methyl-1-penten-3-one	47.40	25044-01-3

*Determined by retention time comparisons of the GC-FID chromatograms.

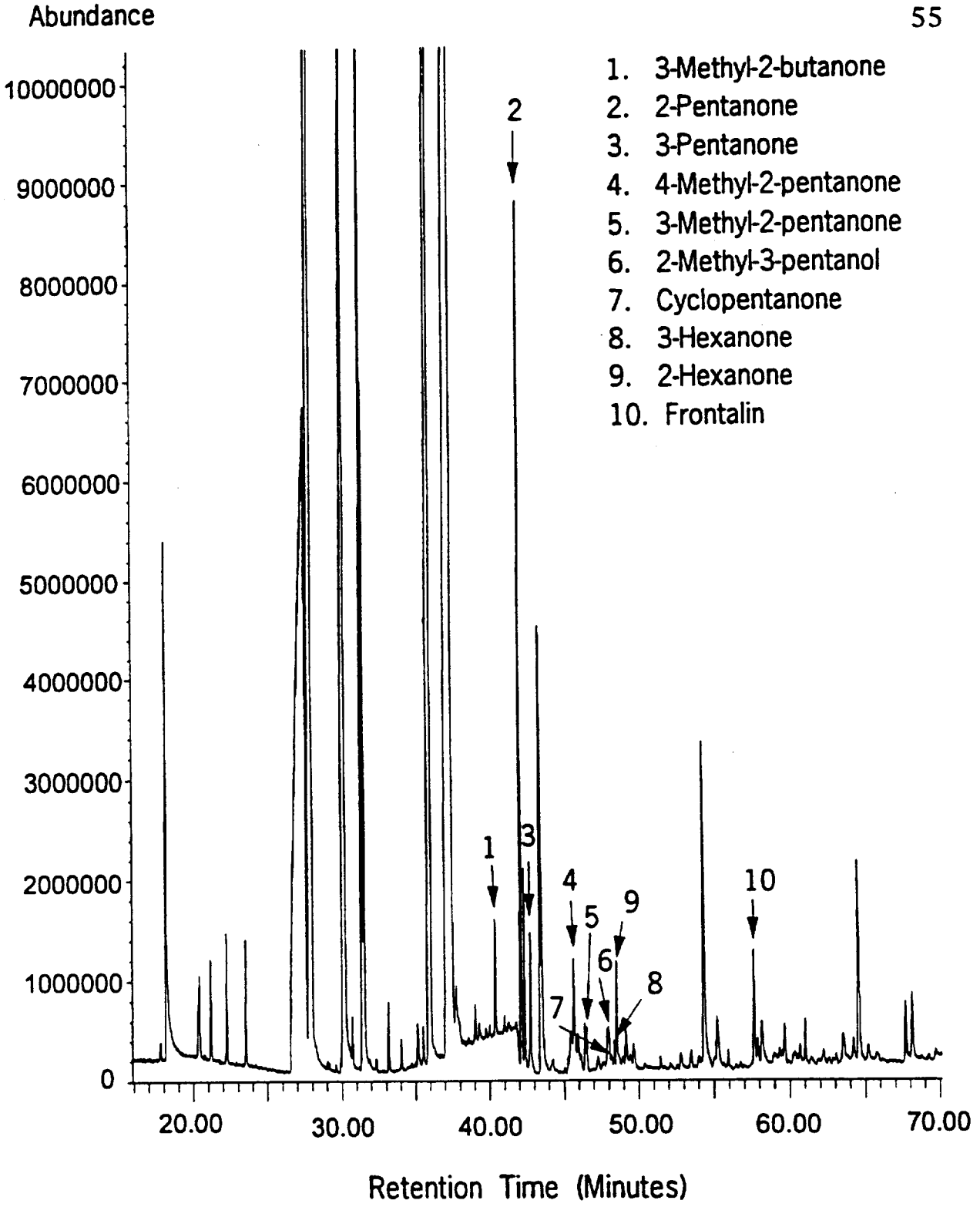

Figure 12. GC-MS total ion chromatogram of the headspace of biologically active TGS. The 10 compounds listed were present only in this bioactive sample and were in an approximate concentration range of 2 ppbv to 130 ppbv.

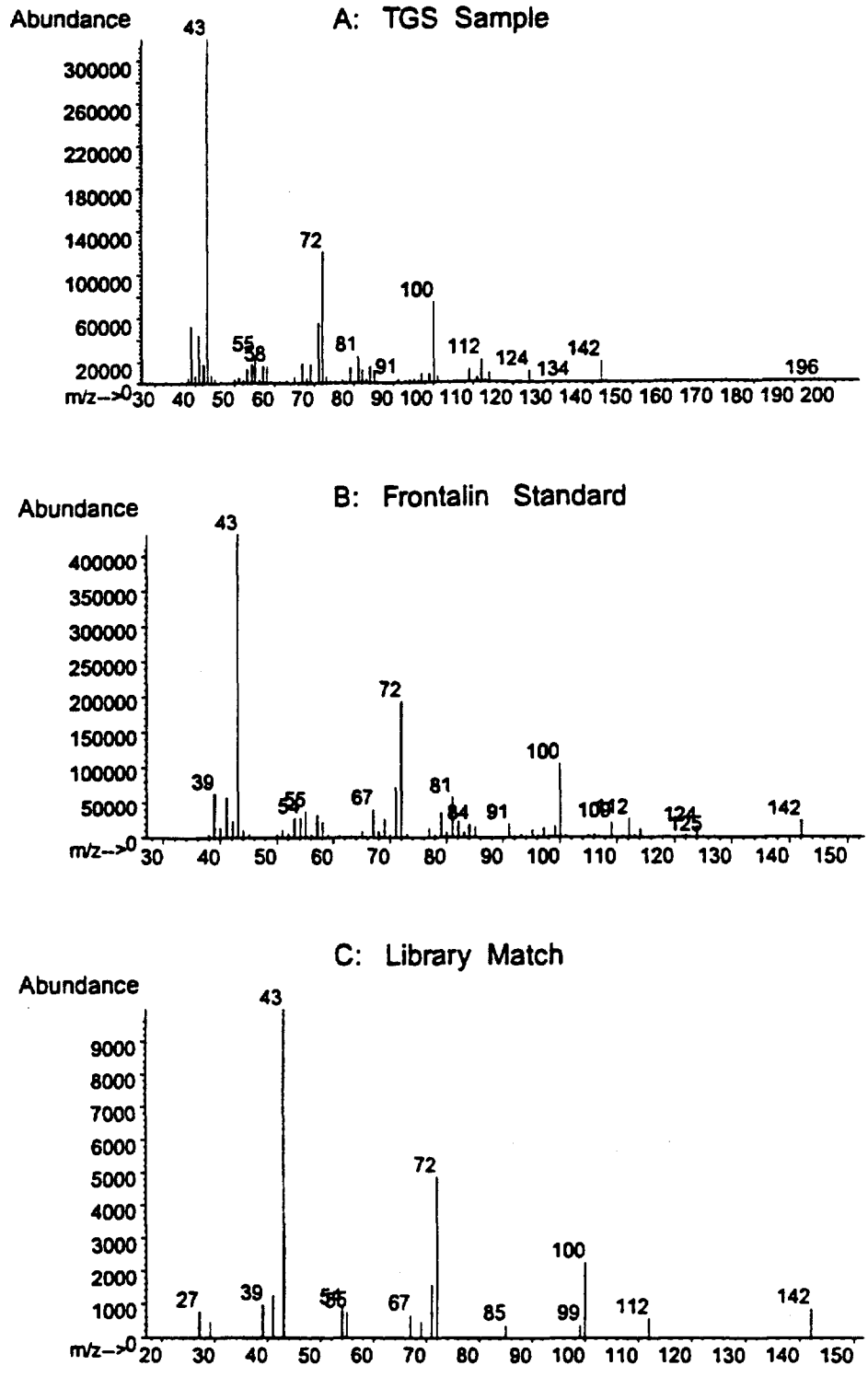

Figure 13. GC-MS spectra of compound in the headspace of pooled bioactive TGS sample (A), a frontalin standard (B), and the frontalin library match (C) from Hewlett-Packard MS ChemStation with a NBS 75K library.

most complex. It was unusual to find this compound in a mammalian secretion. Frontalin has previously been identified only in bark beetles where it acts as an aggregation pheromone (Kinzer et al., 1969; Lindgren, 1992). A comparison of the GC-MS spectra of the compound identified in bioactive TGS, a frontalin standard, and the library match is shown in Figure 13.

Nine compounds were unique to the inactive TGS sample (Table 8). By retention time comparisons of the peaks of the GC-FID chromatograms, it was apparent that one compound, acetic acid, was present in all inactive TGS samples. Perhaps acetic acid plays a role in the inhibition of the avoiding reactions in the females. The GC-MS chromatogram of the pooled inactive TGS headspace sample, with the acetic acid peak indicated, is shown in Figure 14.

The distinct biochemical differences between the headspace of biologically active and in inactive TGS raises several questions. Previous organic extract analyses of TGS show that TGS chemistry changes daily (Rasmussen et al., 1990). Therefore, chemical differences in the headspace of TGS between different sample collections can be expected. Two possibilities for this experimental observation are as follows.

First, TGS may be reflective of the male elephant blood biochemistry. Currently, there is no established evidence that there is a connection between the components in elephant blood and TGS. This avenue is currently being investigated (Perrin and Rasmussen, in preparation). The TGS headspace analyses done for the study presented in this thesis showed high levels of acetone in all TGS samples (Table 6). One characteristic of musth is decreased appetite which leads to significant weight loss. It was hypothesized that the

Table 8. Nine compounds identified by GC-MS that were present only the pooled inactive TGS sample

Compound present in all 10 inactive samples*

Compound	Relative Retention Time (min.)	CAS #
Acetic Acid	43.00-45.00	64-19-7

Compounds present in five or less of the 10 inactive TGS samples*

Compound		
2-Heptanol	55.00	543-49-7
Phenol	58.70	108-95-2

Compounds present in one of the 10 inactive TGS samples*

Compound		
Pentanal	42.40	110-62-3
3-Buten-1-ol	45.10	627-27-0
3-Methyl-2-pentanol	48.70	565-60-6
Hexanal	48.80	66-25-1
3-Hexen-1-ol	52.90	928-96-1
1-Heptanol	58.50	111-70-6

*Determined by comparisons of the GC-FID chromatograms.

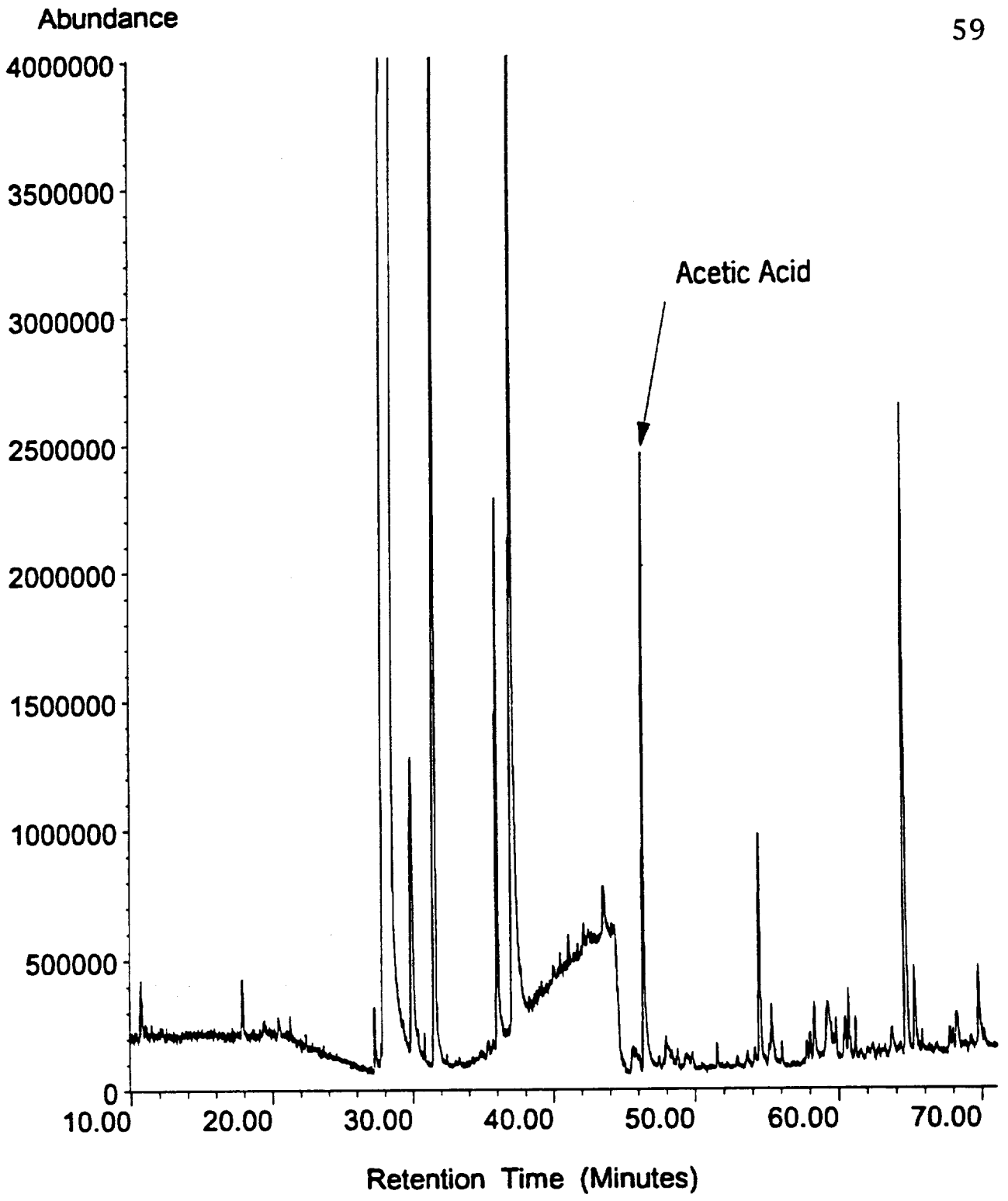

Figure 14. GC-MS total ion chromatogram of the headspace of biologically inactive TGS. Acetic acid, as indicated, was present only in this sample and was at a concentration of approximately 2 ppmv.

presence of acetone in TGS may be resultant from acetone in the blood serum through possible starvation during musth. During starvation, the animal system switches from glucose to ketone bodies as an alternative fuel source. Through ß-oxidation of fatty acids, acetyl-CoA is produced. Through a process known as ketogenesis, acetyl-CoA is converted to the ketone bodies, acetoacetate or D-ß-hydroxybutyrate. The third ketone body, acetone, is produced through nonenzymatic decarboxylation of acetoacetate (Voet and Voet, 1990).

Based on the above hypothesis, weekly concurrent blood, urine and TGS samples will be collected and analyzed from a male Asian elephant (Packy) during his entire musth cycle. If acetone is consistently present in all three sample types, this may indicate a correlation between TGS, blood, and urine biochemistry. This may also present a possible physiological basis for the production of chemosignals in TGS. In this case, TGS chemosignals may be directly resultant from compounds related to starvation or indirectly resultant through by-product formation of compounds related to starvation.

Second, chemical differences between the headspace of successive TGS samples may have resulted from the method in which TGS was collected. TGS samples for this study were taken from the temporal gland by applying pressure to the gland which forced TGS into a beaker. This was an unnatural means of collection because during musth, TGS naturally flows out of the temporal gland down the side of the head of the male elephant. This process may indicate that the temporal gland operates on a physiological cycle. If the temporal gland operates on a cycle, then the TGS collected may not have contained all of the components that would have otherwise been present.

Recently, a new method for TGS volatile collection was developed to circumvent this potential experimental error (Perrin and Rasmussen, in preparation). This method consists of an 850 ml internally electropolished funnel with a Swagelok® fitting connected to an evacuated 850 ml stainless steel sample canister. The funnel-canister apparatus is placed over the temporal gland producing an air tight seal. The valve of the evacuated canister is opened and a headspace sample is taken.

Why do female Asian elephants display avoiding reactions to conspecific male musth temporal gland secretions? During musth, males experience elevated testosterone levels and increased aggression. Males in musth have been known to attack accessible females (Molamure, 1969). Females may cue in on possible aggression-related compounds in TGS. In contrast, perhaps avoiding reactions in females are part of or related to a reproductive scenario. In this case, it may be advantageous for anestrous females to avoid males in musth. This study has led to full-scale experimentation with synthetic compounds, identified in the headspace of TGS, among female Asian elephants. Identification of a compound(s) in TGS that elicits avoiding reactions in females would elucidate possible functions of both female avoiding reactions and male-to-female chemocommunicative aspects of musth.

This initial investigation has demonstrated the effectiveness in using internally electropolished stainless steel canisters to study, at a molecular level, and store biologically active TGS headspace compounds. This method provided substantial information about the concentrations and chemistry of possible TGS avoidance compounds Asian elephants. The success

of this study suggests that the use of the canisters for the study of volatile chemosignals has several advantages. First, once compounds are collected in the canisters, they may be stored, for a reasonable length of time, prior to analyses. This would be advantageous for the collection of samples in the field and/or collection of ephemeral compounds. Second, the volume of sample that can be recovered from the canisters is sufficient to allow multiple analyses. This provides the investigator with the option to experiment with various analytical and bioassay procedures. Third, the sensitivity of the method allows for studies of chemosignals at very low concentrations. The GC-FID and GC-MS systems allowed for the quantification and identification of compounds down to 0.10 ppbv concentration. Lastly, it was demonstrated that the compounds stored in the canisters could be used for bioassays. This was an important feature of the method in that it allowed further testing of the retention of the biologically active compounds well after they were originally collected.

The use of the canisters for experiments with the headspace of TGS was the first application of these canisters to study an animal system. The use of these canisters has already been expanded into studies with other biological fluids of mammals.

CHAPTER IV

GENERAL CONCLUSIONS

The results of this research supported the hypothesis that male-to-female chemosignals exist in musth temporal gland secretion. This study indicated that at least two types of chemosignals are operational in TGS. One, a compound, cyclohexanone, that elicited trunk-to-mouth behavior (i.e., flehmen and PPACR), and the other, a compound(s) that elicited olfactory-mediated avoiding reactions in the females.

Both types of chemosignals may be interrelated. The 10 headspace compounds unique to avoidance eliciting TGS were all within a localized region (40 to 58 minute retention time) of the GC-MS chromatogram. Although not identified in the headspace of TGS, cyclohexanone (retention time = 42 minutes) was also within this localized region. In addition, both types of chemosignals clearly elicited individualistic behavioral response patterns in the females. Individualistic behavior patterns elicited by both types of chemosignals may be related to dominance.

In the future, it will be necessary to establish a behavioral function for both cyclohexanone and possible avoidance compounds. This establishment would necessarily confirm both types of behaviorally and chemically characterized compounds as pheromones in Asian elephants.

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APPENDIX A

BIOASSAY DATA SHEET

Date: Start:
Observer:
# of animals: End:

	Sample #			
Time				
	E1	E2	E3	E4
Totals				

Comments:

Responses: F = Flehmen; PP = Palatal pit; SC = Scrub; C = Check

APPENDIX B

QUANTITATIVE ANALYSIS OF BIORESPONSE SEQUENCES DURING BIOASSAYS WITH CYCLOHEXANONE

Bioresponse sequences have been observed by female and male Asian elephants to naturally occurring exudates and synthetic compounds (L.E.L. Rasmussen, personal communication). These sequences occur in two distinct combinations and are described as follows: 1) A flehmen response is proceeded by a nonindependent check response that expands into a scrub response. This response is summarized as a check » scrub » flehmen response. 2) A PPACR is proceeded by a nonindependent check response that expands into a scrub response. This response is summarized as a check » scrub » PPACR.

Response sequences during both group bioassays and bioassays with Tamba alone occurred often (Table 9). Based on these results, it was hypothesized that flehmen, PPACR, scrub, and check responses are interrelated. These bioresponse sequences may indicate a type of graded chemosensory system. The check response was the most commonly occurring bioresponse during all bioassays. Elephants may utilize the check response as some type of initial chemical screening response. The check response may then expand into a scrub response and then either a flehmen or PPACR.

Currently, only bioresponse sequences by female Asian elephants to cyclohexanone have been monitored. It has been planned to monitor bioresponse sequences during various future bioassays with male and female

elephants. More data would help to establish patterns of the bioresponse sequences and perhaps elucidate their function(s).

Table 9. Summary of bioresponse sequences performed by four female Asian elephants to cyclohexanone

Ten group bioassays

Bioresponse Sequences	% Occurrence Of All Flehmen Responses
Check -> Scrub -> Flehmen Response	50%

	% Occurrence Of All PPACR
Check -> Scrub -> PPACR	37%

Five bioassays with Tamba alone

	% Occurrence Of All Flehmen Responses
Check -> Scrub -> Flehmen Response	100%

	% Occurrence Of All PPACR
Check -> Scrub -> PPACR	67%

APPENDIX C

Table 10. Synthetic compounds bioassayed among eight female Asian elephants at the Metro Washington Park Zoo*

Testosterone
2-Pentanone
5-Nonanone
2-Pentanone-4-hydroxyl, 4-methyl
Acetophenone
6-methyl-5-hepten-2-one
Heneicosanone
4-methyl phenol
Nonylphenol
2-Nonanone
2-Nonanol
3-nonen-2-one
Acetonitrile
2-N-Propylphenol
1-Octen-3-ol
2-Butanone
3-Pentanone
2-Hexanone
2-Heptanone
3-Heptanone
4-Heptanone
2-Octanone
3-Octanone
2-Methylcyclohexanone
3-Methylcyclohexanone
4-Methylcyclohexanone
4-methyl-2-pentanone
Ethanol
Acetic Acid

*These compounds were bioassayed 1-5 times at 20 mM and elicited no response or a novel substance response. Bold print indicates compounds that elicited several check responses.

APPENDIX D

RECENT CYCLOHEXANONE BIOASSAY DATA FROM VARIOUS ELEPHANT FACILITIES

Recently, cyclohexanone has been bioassayed among six captive female Asian elephant groups at the Santa Barbara Zoo, Santa Barbara, California; Bush Gardens, Tampa, Florida; and Dickerson Park Zoo, Springfield, Missouri. The age range of these females was 24-40 years-old. Cyclohexanone was bioassayed at 20 mM concentration 1-2 times, 10 bioassays total, among the groups.

Results showed that persistent and individualistic flehmen, PPACR, scrub, and check responses occurred among all groups tested. When the data was summarized on the basis of dominant versus subordinate elephants, it was apparent that the subordinate females responded to cyclohexanone twice as frequently as the dominant females (Figure 15). This data supported the hypothesis that cyclohexanone has some type of biological meaning to the females and that bioresponse frequency to cyclohexanone is related to dominance hierarchy ranking.

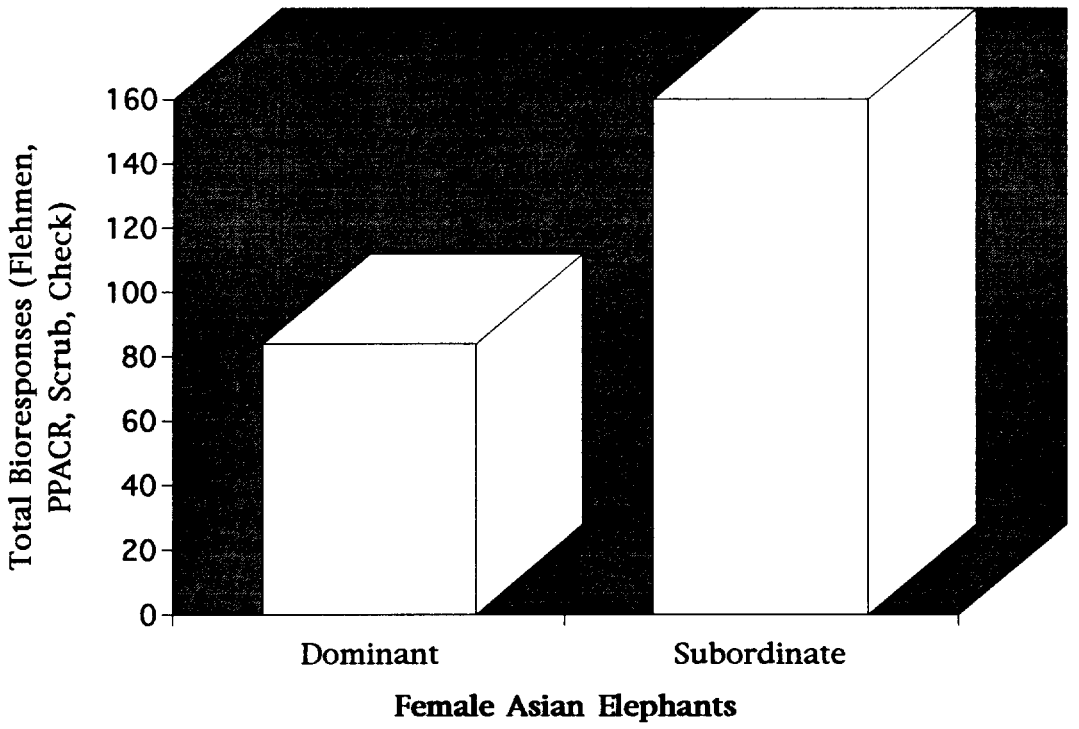

Figure 15. Summary of flehmen, PPACR, scrub, and check responses to cyclohexanone among 21 female Asian elephants during 10 bioassays. Cyclohexanone was bioassayed at 20 mM concentration.

APPENDIX E

SUMMARY OF EAR MOVEMENT AND VOCALIZATION RESPONSES BY FEMALE ASIAN ELEPHANTS TO TEMPORAL GLAND SECRETION

During bioassays of freshly collected TGS and headspace canisters samples of TGS, ear movements and vocalizations (trumpeting, squeaking, and growling) by the females occurred simultaneously with avoiding reactions. These responses were recorded and are reported in Table 11 and 12, respectively. Whether avoiding reactions, ear movements, and vocalizations are interrelated is unknown. These data will be collected during future bioassays with TGS to establish possible patterns of these three responses. This may provide insight into the function of these responses.

Table 11. Summary of backing up, ear movement and vocalization responses of four female Asian elephants to fresh temporal gland secretion samples during 21 bioassays*

A. TGS samples scored as biologically active

	Backed Up				Ear Movement				Vocalizations			
	A	B	C	D	A	B	C	D	A	B	C	D
Sample #												
1	-	+	+	-	+	-	-	-	+	-	-	-
2	x	+	-	x	x	-	-	x	x	-	-	x
3	-	+	+	-	+	-	-	-	-	-	-	-
4	-	+	+	-	-	-	-	-	-	-	-	-
5	-	+	+	-	-	-	-	-	-	-	+	-
6	-	+	+	-	-	-	-	-	-	-	+	-
7	-	+	+	-	-	-	-	-	-	-	-	-
8	-	+	+	-	+	-	-	-	+	-	-	-
9	+	x	x	-	+	x	x	-	-	x	x	-
10	x	+	-	x	x	-	-	x	x	-	-	x
11	x	+	+	x	x	-	-	x	x	-	+	x

B. TGS samples scored as biologically inactive

1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
3	x	-	-	x	x	-	-	x	x	-	-	x
4	-	x	x	-	-	x	x	-	-	x	x	-
5	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-
7	-	x	x	-	-	x	x	-	-	x	x	-
8	-	-	-	-	-	-	-	-	-	-	-	-
9	x	-	-	x	x	-	-	x	x	-	-	x
10	-	x	x	-	-	x	x	-	-	x	x	-

*A,B,C,D = Belle, Hanako, Me-tu, and Sunshine, respectively, and "+" = yes, "-" = no, and x = not present.

Table 12. Summary of backing up, ear movement and vocalization responses among four female Asian elephants to pooled bioactive TGS headspace canister sample stored for one year*

Elephants	Backed Up	Ear Movement	Vocalizations
Belle	-	-	-
Hanako	+	-	+
Me-tu	+	-	+
Sunshine	+	-	-

*There were no responses to pooled inactive TGS or zero air canister samples, and "+" = yes, and "-" = no.

BIOGRAPHICAL SKETCH

The author was born in Tacoma, Washington on June 20, 1968. He has lived in Portland since 1972. In 1990 he graduated from Lewis and Clark College with a B.S. in Biology. From 1987-1990, the author was engaged in an undergraduate internship at the Oregon Graduate Institute of Science & Technology. He began his formal graduate studies at the Oregon Graduate Institute of Science & Technology in 1991.

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