Chem. Senses 28: 433–446, 2003

Frontalin: a Chemical Message of Musth in Asian Elephants (*Elephas maximus*)

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Abstract

Musth is an important male phenomenon affecting many aspects of elephant society including reproduction. During musth, the temporal gland secretions (as well as the urine and breath) of adult male Asian elephants (*Elephas maximus*) discharge a variety of malodorous compounds together with the bicyclic ketal, frontalin. In contrast, teenage male elephants in musth release a sweet-smelling exudate from their facial temporal gland. We recently demonstrated that the concentration of frontalin becomes increasingly evident as male elephants mature. In the present study, we demonstrate that behaviors exhibited towards frontalin are consistent and dependent on the sex, developmental stage and physiological status of the responding conspecific individual. To examine whether frontalin functions as a chemical signal, perhaps even a pheromone, we bioassayed older and younger adult males, and luteal- and follicular-phase and pregnant females for their chemosensory and behavioral responses to frontalin. Adult males were mostly indifferent to frontalin, whereas subadult males were highly reactive, often exhibiting repulsion or avoidance. Female chemosensory responses to frontalin varied with hormonal state. Females in the luteal phase demonstrated low frequencies of responses, whereas pregnant females responded significantly more frequently, with varied types of responses including those to the palatal pits. Females in the follicular phase were the most responsive and often demonstrated mating-related behaviors subsequent to high chemosensory responses to frontalin. Our evidence strongly suggests that frontalin, a well-studied pheromone in insects, also functions as a pheromone in the Asian elephant: it exhibits all of the determinants that define a pheromone and evidently conveys some of the messages underlying the phenomenon of musth.

Key words: chemical senses, 1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane, female elephant behavior, male elephant behavior, pheromone, temporal gland

Introduction

Asian elephants (*Elephas maximus*) rely heavily on their chemical senses to make decisions about foraging and migration, to recognize genetic and social relationships, to identify and choose mates, and to establish and maintain social order (Rasmussen and Krishnamurthy, 2000). Male elephants are an intimate part of the family group as youngsters, but they become increasingly adventuresome (Sukumar, 1989), foraging for a greater variety of fodder farther from the family unit (McKay, 1973). Males mature sexually, from an anatomical perspective, as teenagers (Hildebrandt *et al.*, 2000) but do not mature socially until their twenties, concurrent with regular musth episodes (Jainudeen *et al.*, 1972a,b). As teenagers or before, young males have sporadic, short musth episodes chemically characterized by facial temporal gland emissions of sweet-smelling acetates, alcohols, and ketones, elevated oscillating levels of serum androgens, and periods of erratic behavior (Rasmussen *et al.*, 2002). However, as fully mature adult males, their musth periods lengthen, serum androgen and triglycerides elevate greatly, and their behaviors become overtly aggressive (Rasmussen and Perrin, 1999). Their desire for physically appropriate mates increases. Concomitantly, the chemical composition of the temporal gland secretions (TGS) changes dramatically (Rasmussen *et al.*, 2002).

Male behavior during musth is influenced by internal physiological changes. In turn, the musth behavior affects the physiology and behavior of conspecifics, having ultimate and proximate influences on reproduction. Ultimately, the musth condition may release 'honest' chemical signals and influence the reproductive behavior of females, perhaps via the handicap principle as suggested by Nath (Nath, 1999).

For males, recognition of the ontogenic degree of musth prior to physical encounters is clearly advantageous. Proximately, the musth condition may influence male behavior or may induce reactions by conspecifics, either male or female. For males, immediate reactions are maturity and musth state-related, and include male–male spacing, overt avoidance, or attraction with accompanying flehmen responses to exudates such as urine or temporal gland secretions (Rasmussen *et al.*, 2002). For example, from a proximate perspective, observations in the wild, coupled with instantaneous chemical sampling and captive elephant playback experiments, demonstrated that younger, socially immature males in musth may signal their naïveté by releasing honey-like odors to avoid conflicts with adult males, whereas older musth males broadcast malodorous combinations to deter young males (Rasmussen *et al.*, 2002). These immediate and often lasting effects facilitate the smooth functioning of male society.

A similar duet of proximate and ultimate exchanges of chemical signals resulting in altered or informed behaviors between musth males and females may also be operational. During controlled playback studies, some female elephants avoided and retreated from many, but not all, fresh mature male musth TGS samples, whereas only a few freezer-stored (–20°C) samples were effective (Perrin *et al.*, 1996). Since only subordinate females exhibited the retreat behaviors, the tested population was too small to reach a meaningful conclusion, although dominance appeared to be a factor. Concurrent chemical analyses demonstrated that a specific complement of ketones and alcohols and a bicyclic ketal compound were always present in the samples that elicited bioresponses (Perrin *et al.*, 1996).

Since the ketonic compounds elicited variable behavioral responses, we focused our attention on the bicyclic ketal, frontalin (1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane) (Figure 1), a well-characterized bark beetle pheromone (Kinzer *et al.*, 1969; Phillips *et al.*, 1990; Lindgren, 1992). In the Asian elephant, frontalin is present in the TGS, urine, and breath of mature males in musth (Rasmussen, 1998). However, frontalin is absent in the TGS of subadult male elephants experiencing their first or moda musths, only becoming detectable in the TGS as the males mature and experience successive, more adult-like musth episodes (Rasmussen *et al.*, 2002). Bioassays of whole TGS with male elephants and observations in the wild of male–male behavior demonstrated that older males are somewhat indifferent to moda musth males and their secretions (which lack frontalin), while younger adolescent males avoid TGS and older males in musth (Rasmussen *et al.*, 2002). Thus, there is a congruence of chemical and behavioral events evident in male–male interactions.

Initial tests also suggested that female elephants respond differently to TGS containing or lacking frontalin (L.E.L. Rasmussen, unpublished data). The documentation of distinctive chemosensory responses and behaviors toward frontalin by males and females would elucidate whether frontalin plays a role as a chemical signal in reproduction. Such information should allow elephants to recognize male musth state prior to physical encounters and to adjust behavioral responses appropriately. Therefore, we hypothesized that Asian elephants recognize this male-emitted compound chemosensorily, employing it to assess the sexual maturity and/or musth state of male conspecifics, and that this recognition would be evident in behavioral reactions and responses that maintain social order and hierarchy. To test our hypotheses, we designed a series of bioassays to assess the behavioral and chemosensory responses of elephants of both sexes to synthetic frontalin. We present the results and interpretation of these experiments, analyzed from the perspective of the sex, maturity, and hormonal status of the tested elephants that strongly implicate frontalin as having a distinct chemical messaging functionality in Asian elephants.

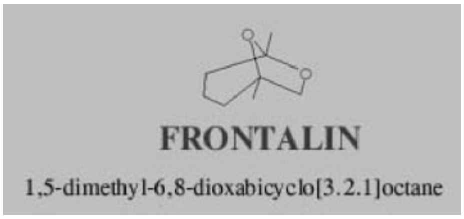

Figure 1 Structure of frontalin.

Materials and methods

Elephants and facilities

A total of 30 elephants, 21 females and nine males, were available for this study. The elephants were housed at Riddle's Elephant Sanctuary (RES), Greenbrier, AR ($n = 4$), the Ringling Center for Elephant Conservation (RCEC), Polk City, FL ($n = 23$), and the Oregon Zoo, Portland, OR ($n = 3$). Each facility had a sizable enclosure yard available for bioassays.

All bioassays with male elephants were conducted on nonmusth animals. Nonmusth was defined by behavioral characteristics and physiological parameters including serum testosterone concentrations below 10 ng/ml (Rasmussen and Perrin, 1999). Among males, two categories of sexual maturity were recognized: (i) subadults, i.e. sexually mature but socially immature teenage or subteenage males, $n = 3$, and (ii) older adults, i.e. fully socially and sexually mature males, ranging in age from 27 to 34 years, $n = 6$.

The female elephants were all sexually mature. All were cycling except four pregnant elephants. The hormonal status of the 17 cycling females was ascertained after the bioassays were completed. Serum concentrations of the steroid hormone, progesterone, were then used to categorize the females prior to data analysis.

Hormonal data

Serum hormone concentrations (testosterone in males and progesterone in females) were measured by radioimmunoassay by Dr David Hess (Oregon Health & Science University, Beaverton, OR), Dr Thomas Goodwin (Hendrix College, Conway, AR), and by Ringling Center for Elephant Conservation (Polk City, FL). Sensitivity for testosterone and progesterone levels was 10 and 15 pg/tube, respectively, at 90% binding levels using validated assays (Resko *et al.*, 1973, 1980; Hess *et al.*, 1983; Rasmussen *et al.*, 1984).

Bioassays

The bioassay scheme has been detailed previously (Rasmussen *et al.*, 1982, 1986, 1996). The present investigation was conducted as a double-blind study, i.e. the investigator was not aware of the identity of the samples, and the elephant did not see the samples being placed. Two samples were randomly placed on ground-level concrete areas, at least 20 feet apart, prior to the entry of the test elephant(s). Temperature, moisture, and wind speed and direction were recorded. Elephants were observed for 1 h from a distance adequate to see clearly but non-distracting to the animal. All occurrences of standard chemosensory responses and evoked behaviors described below were recorded for the focal animal(s) using simultaneous voice and video recording. All assays with males were conducted on solitary individuals. Individual males were repetitively tested, a total of six times.

All females were assayed as part of a group, not as solitary subjects. Between one and 10 tests, each separated by 3–6 month intervals, were conducted on 21 female elephants over a period of 5 years. Females were either assayed one time only in their luteal, follicular or pregnant stages, or they were assayed repetitively, for a total of five times each in the luteal and follicular phases or three times each while pregnant. Except for the pregnant elephants, the estrous status was unknown to the investigator at the time of the assay. Since the estrous cyclicity varied among females, single assays of females in either their luteal or follicular phase were easily obtained. The testing of multiple-assayed females was randomized between the luteal and follicular phases by varying the number of months between bioassays. After the assays, the data from the cycling females were sorted into two reproductive categories, based on serum progesterone concentrations: follicular (F) or luteal (L).

Responses and behaviors

Scored olfactory-implicated responses included sniffs, trunk shakes, blows (forceful exhalations), checks, places, sucks and flehmens. The data were expressed as responses per hour. During data analyses the responses were categorized as main olfaction (sniffs, trunk shakes and blows), pre-flehmen (checks, places and sucks) and flehmen. Sniffs by Asian elephants are clearly delineated by overt directionality of the trunk tip, slight compression of trunk musculature, and sometimes-audible inhalation, with involvement of the main olfactory system being assumed. Trunk shakes are mild exhalations that occur subsequent to sniffs and some pre-flehmen responses. Blows are forceful exhalations of air through the trunk. Both of these latter responses are presumably for the purpose of clearing odorants from the truncal passages. In the pre-flehmen response, the check, only the trunk tip finger is placed in the sample area; slight trunk compression is often noticed and inhalation may be heard. The place response involves the placement of the entire trunk tip flush with the sample area on the ground. During a suck, the entire trunk tip is also flush with the substrate and there is accompanying trunk compression, often with audible inhalation. The main olfactory system is postulated to be at least partially involved in the three pre-flehmen responses (Rasmussen and Schulte, 1998). During the flehmen response, the trunk tip finger containing drops of mucus accompanied often with urine or sample is placed precisely on the openings of the vomeronasal duct on the dorsal anterior palate; the vomeronasal organ is implicated in this response (Rasmussen *et al.*, 1982; Lazar *et al.*, 2002).

The recorded evoked behaviors included avoidance (circling motions around samples with no contact), repulsion (backing up or moving away from the sample after chemosensory responses such as main olfactory, pre-flehmen or flehmen responses), vocalizations (trumpeting and roaring), and conspecific palatal pit responses. Palatal pit responses are characterized by an elephant placing its trunk tip into the palatal pits, small hair-filled crypts located in the oral mucosa lateral to the vomeronasal organ openings (A. Hansen and L.E.L. Rasmussen, unpublished data). Placement may be to the elephant's own palatal pits or to those of a conspecific. Sender and receiver elephants were identified for the latter responses. Indifference to samples was also recorded. The sequences of chemosensory responses and evoked behaviors were recorded as the total number of occurrences during the bioassay hour.

Samples

In each assay, paired samples were presented: 100 μM frontalin in 0.01 M phosphate buffer, pH 8.0, and buffer control. (+/–)-Frontalin was purchased as a racemate from Pherotech (Delta, BC, Canada) and used without further purification. We estimated the physiologically reasonable test concentration (100 μM) based on levels of frontalin measured in natural temporal gland volatiles (Perrin *et al.*, 1996; Rasmussen *et al.*, 2002).

When studying captive elephants, there are logistical challenges and unforeseen variables; experimental conditions are sometimes less than ideal compared with small mammals. The cognitive, olfactory-oriented elephant is curious about novel odors and mixtures. Extensive experience in elephant testing has demonstrated the necessity for repetitive testing to sort out responses to novel mixtures from responses to biologically meaningful samples (Rasmussen *et al.*, 1986). With valid chemical signals or pheromones, responses do not diminish over time in mature elephants of constant hormonal status, although some variation between individual elephants may be observed. We are aware of the problem of pseudoreplication. Because of the novel substance response phenomenon, we deliberately tested selected elephants, both male and female, six and five times, respectively, over several years to include the females' luteal and follicular estrous states. The data were analyzed from several perspectives and groupings. In Tables 1, 4, 8 and 9, the analyses from the first bioassays are presented. In Tables 2, 5 and 10, repetitive test data are presented. We present ranges and averages so that attributes of the individual assays are more evident. Tables 3 and 7 detail the behavioral sequences.

Data analysis

Male elephants were categorized by maturity status and age, whereas female elephants were grouped according to reproductive status: luteal phase, follicular phase or pregnant. Because part of this study involved repeated measures of the same individual males and females, these measurements are not independent and tests such as the Mann–Whitney Rank Sum are only applicable when first assays are compared. In addition, the data included zeros, which presents another issue, necessitating statistical restraint. For the males, we tested three subadult males six times, and six adult males six times. Initially, only the first assay results of the three subadult and the six older males were analyzed by the Mann–Whitney Rank Sum test (Table 1). Subsequently, the response frequencies were compared for repetitively tested younger and older males (Table 2). The data are listed as ranges, averages, and medians; repeated measures of one-way analysis of variance (ANOVA) and pair-wise comparisons with the Student–Newman–Keuls test were conducted (Tables 1 and 2).

The study of the 21 female elephants included those in three hormonal phases: follicular, luteal, and pregnant. We hypothesized that females in the follicular phase would be more responsive to the musth state of the male as revealed by frontalin. We assayed six female elephants in the luteal phase one time only and six different females in the follicular phase also one time only. The data were tabulated and statistically compared using the Mann–Whitney Rank Sum test (Table 4).

We wanted to confirm, by repetitive testing, the data from the first tests. We hypothesized that if frontalin was a valid reproductive chemical signal (i.e. a pheromone), operational from male Asian elephants to conspecific females, then females would also respond more during their follicular rather than their luteal phase. Balanced data sets were obtained for five different female elephants, repetitively tested five times each in their follicular and luteal phases (Tables 5 and 6). We tested this confirmatory hypothesis through the use of repeated measures of ANOVA using Statistica (StatSoft, Inc.). Since we had the required balanced data sets, repeated measures analysis avoided some pseudoreplication issues (Schulte and Rasmussen, 1999). During ANOVA analyses of the repetitive female data, the phase in estrus was the independent variable while the sample type (frontalin versus buffer) and the tests (1–6) were the repeated measures. We only statistically compared main olfactory and pre-flehmen responses between luteal- and follicular-phase females (Table 7), because luteal-phase females exhibited no flehmen responses (Table 6). We report the data for flehmen responses, but because of the zeros in the flehmen data, we focused on the main olfactory and pre-flehmen responses.

We postulated that pregnant elephants, perhaps in critical stages of pregnancy, need to be aware of the state of the male. Our data from pregnant elephants are less comprehensive than for cycling females. Testing was somewhat more opportunistic and more variables were present. For example, assays were conducted at varying times during the 22 month gestation period. As a first test, we were able to assay four pregnant elephants in varying stages of pregnancy (Tables 8 and 9). Three elephants, including two that were assayed first during their non-pregnant state, were repetitively assayed three times in the pregnant state (Table 10).

Results

The responses by male and female elephants to the buffer only were limited to an occasional sniff or check when presented paired with 100 μM frontalin buffer in each assay. Therefore only the data from responses to frontalin are presented.

Males

Individual males were tested six times with placed samples of 100 μM frontalin, paired to buffer controls. The response data were analyzed in terms of first tests and all tests (Table 1). The first assays with the younger males demonstrated high and tightly distributed chemosensory responses, including main olfactory (MO) responses, pre-flehmen responses and flehmens (Table 1). In contrast, male Asian elephants in the fully mature category (ages 26–38 years) responded at very low levels to frontalin. Except for one older, subdominant male (OA1), older males only responded with a few sniffs and checks that were randomly scattered among the assays. The data from the first bioassays with the younger subadult males were compared with those of the older males and analyzed by Mann–Whitney Rank Sum tests. Statistical tests demonstrated that the three subadult males (SA1–SA3) performed significantly greater numbers of pre-flehmen and flehmen responses to frontalin than the six older males (OA1–OA6)

Table 1 First presentation trials: comparative responses by subadult[a] and older[b] male Asian elephants to frontalin[c] [values are medians (25–75 percentiles)]

Response/h	Subadult males	Older adult males
Main olfaction[d]	4.0 (3.25–4.00)	1.0 (1.0–2.0)
Pre-flehmen[e] (*SSD)[f]	9.0 (7.5–11.25)	1.5 (0.0–2.0)
Flehmens (*SSD)[f]	2.0 (2.0–2.75)	0 (0.0–1.0)

[a] Subadult males (SA), ages 7–13 years, were sexually mature but not socially mature, $n = 3$.

[b] Older males (OA), ages 26–38 years, $n = 6$.

[c] Frontalin (1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane) as liquid presentation in 0.01 M phosphate buffer, pH 8.0, was assayed at 100 µM.

[d] MO response categories include sniff, trunk shake and blow responses and involve primarily the main olfactory system.

[e] Pre-flehmen responses included check, place and suck responses.

[f] Mann–Whitney Rank Sum test, *U*-test statistically significant difference (*SSD) between SA and OA, $T = 22$, $P \leq 0.02$.

(Table 1), whereas no significant differences were observed in the main olfaction (MO) categorized responses (Table 1).

Repetitive testing generally confirmed the results seen in the first tests, i.e. that the subadult males performed significantly more flehmen and pre-flehmen responses to frontalin than the older males, but there was little difference in MO responses between the two groups (Table 2). However, as repetitive testing allowed more extensive comparison of individual males, some individual differences were revealed. Older males rarely exhibited flehmen; the exception was older male OA1 whose flehmen response frequency was not statistically different from SA2 and SA3. Also, subadult male SA1 demonstrated significantly greater flehmen responses than all six older males and SA2 and SA3 (Table 2). All older adults, except OA1, showed significantly reduced pre-flehmen responses compared to those of all subadult males but with varying degrees of significance (Table 2). Older male OA1 performed pre-flehmen responses that were significantly different from subadult male SA1 and SA2, but not SA3. One older male OA4 was sufficiently non-responsive during repetitive testing that its olfactory categorized responses were significantly lower than those of all subadult males. Figure 2 summarizes the response differences between subadult and older males as well as between groups of females.

Sequential chemosensory–behavioral responses by male elephants after sampling the placed frontalin samples were sorted into three categories: attraction, indifference, and repulsion/avoidance (Table 3). Three younger males (ages 7–13 years), tested over several years, demonstrated strong reactions of backing up (repulsion) and avoidance (circling around) to frontalin samples with accompanying roaring and trumpeting vocalizations. The older males exhibited indifference in correlation with chemosensory responses and exhibited no vocalizations or aversive movements (Table 3).

Females

Serum progesterone (P_4) concentrations for the only-once-tested females ranged between undetectable and 92 pg/ml for the six follicular-phase females (A, D, H, I, M and O), and between 340 and 1294 pg/ml for the six different luteal-phase females (C, E, F, N, U and Y) (Table 4). Five of the 21 females (B, J, P, S and T) were repeatedly tested in five assays during the follicular phase, and the same five females were repeatedly tested five times during the luteal phase (Tables 5 and 6). Their P_4 concentrations were in the ranges 6–62 pg/ml and 404–1069 pg/ml, respectively. Repetitive testing demonstrated that responses to frontalin were robust. There was no evidence of novel substance response (Rasmussen *et al.*, 1986).

First tests of six follicular-phase females and six different luteal-phase females demonstrated that MO and pre-flehmen responses by follicular-phase females were significantly higher than those by luteal-phase females as seen by averages, ranges and medians, and confirmed by Mann–Whitney Rank Sum test (Table 4). Flehmens were not significantly elevated, but the lack of response such as the nil responses by luteal-phase females made statistics difficult; thus statistical restraint was applied. Flehmens and longer pre-flehmen responses (i.e. place and suck responses) were elicited in more than half of the follicular-phase females, whereas such responses were not seen with females tested in their luteal phase (Table 4).

Responses tabulated from repetitive testing of five different females (B, J, P, S and T) during their follicular and luteal phases were statistically compared. First, however, we tested both the data collected during the follicular phase and that collected during the luteal phase for intra-phase differences by one-way repeated measures of ANOVA and then pair-wise multiple comparisons using the Student–Newman–Keuls test. For MO responses, no intra-phase differences were observed for either follicular or luteal phases [$\chi^2 = 36.5$ with nine degrees of freedom (d.f.), probability (P) < 0.001; difference of ranks (DR) = 13.5–39.5, $q = 4.42$–8.56]. For pre-flehmen responses, no differences were observed during the follicular phase; however, several significant intra-luteal differences were noted ($\chi^2 = 41.0$ with d.f. = 9, $P < 0.001$; DR = 12.0–35.0, $q = 4.50$–9.84). Flehmen data revealed many non-responders; these zeros precluded vigorous testing, but no significant differences were revealed ($\chi^2 = 20.0$ with d.f. = 9, $P = 0.018$).

Subsequently, the responses of individual females during their luteal phase were compared with those during the follicular phases (Table 7). As analyzed by repeated measures of ANOVA on ranks followed by pair-wise comparison using the Student–Newman–Keuls procedure for all five females with five repetitive tests in each phase, all female

Table 2 Repetitive[a] bioassay trials: responses per hour [medians (25–75 percentiles)] to 100 μM frontalin[b] by individual subadult and older adult nonmusth Asian male elephants

Age at test	Main olfactory[c]	Pre-flehmen[d]	Flehmen
Subadults[e]			
SA1 (7–9 y)			
Range	3–6	3–28	1–5
Average	4.5	16.3	2.8
Median (25–75)	4.5 (4.00–5.00)	16.50 (12.00–22.00)	3.00 (1.00–4.00)
*SSD[f]	*OA-4[f]	*OA1–6[f]	SA 2–3
SA2 (10–12 y)			
Range	2–5	2–9	0–3
Average	3.3	5.2	1.5
Median	3.50 (2.00–4.00)	5.00 (4.00–6.00)	1.50 (1.00–3.00)
*SSD[f]	*OA-4[f]	*OA1–6[f]	*OA2–6[f]
SA3 (10–13 y)			
Range	2–4	2–7	1–3
Average	2.7	3.2	1.3
Median	2.50 (2.00–3.00)	3.50 (3.00–5.00)	1.00 (1.00–1.00)
*SSD[f]	*OA-4[f]	*OA2–6[f]	*OA2–6[f]
Older adults[g]			
OA1 (28–30 y)			
Range	1–3	1–12	0–5
Average	2.0	4.3	1.0
Median	2.00 (1.00–3.00)	3.00 (1.00–6.00)	1.00 (0.50–1.00)
OA2 (29–31 y)			
Range	0–3	0–2	0[h]
Average	0.83	0.5	0
Median	0.50 (0.00–1.00)	0.00 (0.00–1.00)	0
OA3 (36–38y)			
Range	1–4	0–2	0–1
Average	1.5	1.16	0.16
Median	1.00 (1.00–1.00)	1.50 (0.0–2.00)	0
OA4 (36–38 y)			
Range	0–1	0–1	0
Average	0.33	0.16	0
Median	0 (0.00–1.00)	0 (0–0)	0
OA5 (33–37 y)			
Range	0–2	0	0
Average	0.83	0	0
Median	1.00 (0.00–1.00)	0	0
OA6 (26–28 y)			
Range	1–3	0–4	0
Average	1.33	0.66	0
Median	1.00 (1.00–1.00)	0	0

*SSD = statistically significant difference.

[a] Six tests at 6 months intervals.

[b] Frontalin (1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane) as liquid presentation in 0.01 M phosphate buffer, pH 8.0, was assayed at 100 μM.

[c] MO response categories include sniff, trunk shake and blow and involve primarily the main olfactory system.

[d] Pre-flehmen (PreF) responses included check, place and suck responses.

[e] Subadult males (SA), ages 7–13 years, were sexually mature but not socially mature, $n = 3$.

[f] Repeated measures ANOVA followed by pair-wise comparison (Student–Newman–Keuls test). For significant differences, $P = 0.001$, $\chi^2 = 28.456$ (MO), 40.089 (PreF), 40.433 (Flehmen), difference of ranks (DR) varied from 21.0 to 43.50 and q from 3.13 to 6.49.

[g] Older males (OA), ages 26–38 years, $n = 6$.

[h] Zero responses rendered certain statistical tests technically invalid.

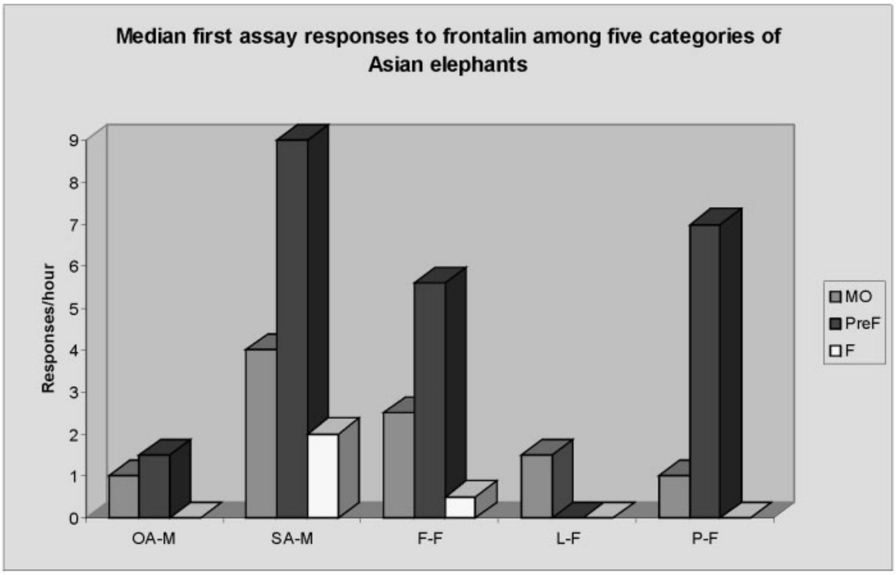

Figure 2 Median response frequencies during first presentations of 100 μM frontalin. Responses were categorized as main olfactory (MO), pre-flehmen (PreF) and flehmen (F). Five groups of Asian elephants were compared: older adult males (OA-M), subadult males (SA-M), females in the follicular phase of estrus (F-F), females in the luteal phase of estrus (L-F), and pregnant females (P-F). Details of responses and statistics are in Tables 1–11.

Table 3 Sequential behavioral responses by subadult Asian elephant males following recorded distinct chemosensory responses to frontalin

Chemosensory response	Followed in sequence by	*n*
Flehmen →	roar	2
Sniff →	back-up	2
Check → trunk shake	back-up	2
Sniff → trunk shake	back-up	1
Check → trunk shake	block female access to frontalin	1
Check → blow	roar	1
Place → blow	ran away from	1
Sniff →	circled around	1
Sniff →	avoid	1
Sniff →	trumpet	1

n = number of observations.

elephants in the follicular phase exhibited a significantly higher frequency of chemosensory responses than females in the luteal phase for the MO and pre-flehmen responses (Table 7). No flehmens were observed to frontalin during the luteal phase (Table 6); however, one or two flehmens per session were often observed during the follicular phase (Table 5).

The four pregnant females tested for the first time with frontalin demonstrated statistically significant fewer MO responses (Table 8) than did the follicular-phase females (Table 9). The first tested pregnant females showed no flehmen responses (Table 8). Pre-flehmen responses for first-tested pregnant females were not different from responses from the single-tested follicular-phase females, but were

Table 4 First assays: comparative responses by luteal- and follicular-phase female Asian elephants, responses per hour to 100 μM frontalin

Female	Main olfactory[a]	Pre-flehmens[a]	Flehmen[b]
Females in follicular phase (P_4 serum concentrations: not detected–92 pg/ml)			
A	3	6	0
D	4	5	1
H	3	6	1
I	4	4	1
M	3	5	0
O	4	12	0
Range	3–4	4–12	0–1
Average	3.7	6.3	0.5
Median[c]	3.5 (3.0–4.0)	5.6 (5.0–6.0)	0.5 (0–1.0)
Females in luteal phase (P_4 serum concentrations: 340–1294 pg/ml)			
C	2	0	0
E	0	0	0
F	2	0	0
N	0	0	0
U	1	1	0
Y	2	0	0
Range	0–2	0–1	0
Average	1.1	0.16	0
Median[c]	1.5 (0.0–2.0)	0 (0–0)	0 (0–0)

[a] Statistically significant differences, Mann–Whitney Rank Sum test, $P = 0.001$, $T = 21$.

[b] No significant difference, $P = 0.09$.

[c] Values are medians (25–75 percentiles).

Table 5 Repetitive tests: tabulated chemosensory and behavioral responses per hour to 100 μM frontalin in follicular-phase Asian elephant females (*n* = 5) (P_4 = 6–62 pg/ml)

Female	Test no.	Main olfactory	Pre-flehmen	Flehmen
B	2	2	3	0
B	3	6	24	1
B	4	4	10	0
B	7	3	10	0
B	10	7	8	0
Range		2–7	3–24	0–1
Average		4.4	11.00	0.2
Median		4.5 0 (2.75–6.25)	10.00 (6.75–13.50)	0.00 (0.00–0.25)
J	1	4	16	1
J	4	4	10	0
J	6	4	9	1
J	8	3	5	0
J	9	4	8	0
Range		3–4	5–16	0–1
Average		3.8	9.6	0.5
Median		4.00 (3.75–4.00)	9.00 (7.25–11.50)	0.00 (0.00–1.00)
P	2	4	2	0
P	3	9	16	1
P	5	6	6	0
P	8	7	4	0
P	9	6	5	0
Range		4–9	2–16	0–1
Average		6.4	6.6	0.2
Median		6.00 (5.50–7.50)	5.00 (3.50–8.50)	0.00 (0.00–0.00)
S	2	5	12	1
S	3	3	5	2
S	5	2	5	1
S	7	3	5	0
S	10	3	4	0
Range		2–5	4–12	0–2
Average		3.2	6.2	0.8
Median		3.00 (2.75–3.25)	5.00 (4.75–6.75)	1.00 (0.00–1.25)
T	2	3	5	1
T	4	3	5	1
T	6	3	8	1
T	7	4	5	0
T	10	2	4	0
Range		2–4	4–8	0–1
Average		3.0	5.5	0.6
Median		3.00 (2.75–3.25)	5.00 (4.50–5.75)	1.00 (0.00–1.00)

significantly greater than those from luteal-phase females (Table 9). Likewise, the three triple-tested pregnant females (Table 10) demonstrated significantly higher levels of pre-flehmen responses than females in the luteal phase (d.f. = 33, $P < 0.001$, F = 12.5–29.9). Statistically, the triple-tested pregnant females demonstrated lower flehmen responses than follicular phase females (d.f. = 33, $P = 0.05$, $F = 4.25$).

Some noteworthy behavioral sequences following chemosensory responses were seen among follicular- and luteal-phase and pregnant females (Table 11). The sequential behaviors varied somewhat, depending on the estrous state or pregnancy. On two occasions, luteal-phase females demonstrated avoidance of frontalin by circling the sample. Pregnant females were considerably more apprehensive; during one of the two rumbling vocalization sessions, a

Table 6 Repetitive tests: tabulated chemosensory and behavioral responses per hour to 100 μM frontalin in luteal-phase female Asian elephants from Table 5 (P_4 = 404–1069 pg/ml)

Female	Test no.	Main olfactory	Pre-flehmen	Flehmen
B	1	2	0	0
B	5	1	0	0
B	6	2	0	0
B	8	1	1	0
B	9	2	0	0
Range		1–2	0–1	0
Average		1.6	0.2	0
Median		2.00 (1.00–2.00)	0.00 (0.00–0.25)	0.00 (0.00–0.00)
J	2	2	0	0
J	3	1	0	0
J	5	1	0	0
J	7	2	0	0
J	10	2	0	0
Range		1–2	0	0
Average		1.6	0	0
Median		2.00 (1.00–2.00)	0.00 (0.00–0.00)	0.00 (0.00–0.00)
P	1	0	0	0
P	4	0	0	0
P	6	0	0	0
P	7	1	0	0
P	10	1	0	0
Range		0–1	0	0
Average		0.2		
Median		0.00 (0.00–1.00)	0.00 (0.00–0.00)	0.00 (0.00–0.00)
S	1	2	0	0
S	4	0	0	0
S	6	0	0	0
S	8	1	1	0
S	9	1	0	0
Range		0–1	0–1	0
Average		0.8	0.2	0
Median		1.00 (0.00–1.25)	0.00 (0.00–0.25)	0.00 (0.00–0.00)
T	1	1	1	0
T	3	3	1	0
T	5	2	1	0
T	7	2	1	0
T	10	1	1	0
Range		1–3	1	0
Average		1.8	1.0	0
Median		2.00 (1.00–2.25)	1.00 (1.00–1.00)	0.00 (0.00–0.00)

female urinated and then performed a palatal pit contact response to a conspecific. Characteristically, during all but one assay of the pregnant elephants, palatal pit responses (either to self or to a conspecific) were observed. During one multiple palatal pit sequence, the initiator elephant checked the temporal gland of a conspecific (Table 11). The follicular-phase females were chemosensorily responsive, spent extended periods at frontalin samples, and performed penile and temporal gland checks on male elephants.

Discussion

This paper presents evidence from bioassays of synthetic frontalin with conspecific Asian elephants demonstrating that frontalin is a putative pheromone in this species.

Table 7 Statistical comparison[a] of responses per hour to 100 µM frontalin by five female Asian elephants tested during both their follicular and luteal phases

	SSD follicular versus luteal	T	$P <$
MO			
B	Yes	38.5	0.01
J	Yes	40.0	0.008
P	Yes	40.0	0.008
S	Yes	39.5	0.008
T	No		
Pre-flehmen			
B	Yes	40.0	0.008
J	Yes	40.0	0.008
P	Yes	40.0	0.008
S	Yes	40.0	0.008
T	Yes	40.0	0.008
Flehmen			
B–T	No		

SSD = statistically significant difference.

[a] One-way repeated measures of ANOVA and then pair-wise multiple comparison using Student–Newman–Keuls test.

Table 8 First tests: pregnant female Asian elephants (P_4 = 772–1026 pg/ml)

Females (pregnancy length)	Responses/hour		
	Main olfactory	Pre-flehmen	Flehmen
L (9 months)	1	12	0
K (1 month)	1	6	0
G (4 months)	2	8	0
Q (3 months)	1	3	0
Range	1–2	3–12	0
Average	0.83	7.25	
Median[a]	1.00 (1.00–1.50)	7.00 (4.50–10.00)	0.00 (0.00–0.00)

[a] 25–75 percentiles.

Frontalin is released from a specific gland (the temporal gland) of male elephants during a specific physiological state (musth) and carries an intraspecies message to conspecifics, eliciting immediate chemosensory and behavioral responses. A differential spectrum of chemosensory responses and immediate, subsequent behavioral reactions by male and female elephants support the reception of a finely tuned chemical message. Tests with subadult and adult male and female African elephants evoke only sniffs and exhalations and no overt behaviors (T.E. Goodwin, B.A. Schulte and L.E.L. Rasmussen, unpublished data).

Pheromones are further defined as contributing 'to evolutionary fitness or mutual benefit of both sender and receiver' (Meredith, 2000). Frontalin fulfills this definition in the Asian elephant. The frontalin chemosensory message from the musth temporal gland of older, more experienced, often dominant male elephants gives a clear indicator to male (and female) conspecifics of the sender's sexual state and social maturity. Other males of equal status can choose to ignore such signals or on rare occasions may engage in combat. Younger, smaller males, not releasing frontalin themselves, can avoid costly fights with older, larger, more experienced males (Rasmussen *et al.*, 2002). Females can assess maturity, physiological condition, and reproductive fitness as older males stay in musth longer and secrete more frontalin. Elevated chemosensory responses by follicular-phase females and the lack of response by luteal-phase females reflect differential assessments that mutually benefit male senders and female receivers. Apprehensive reactions and elevated chemosensory responses by pregnant females indicate their recognition of the advantage of not encountering aggressive musth males.

Frontalin as a pheromone also imparts important short-term social and reproductive messages that elicit immediate and evidential reactions and behaviors from both sexes. Older males exhibit awareness of frontalin by sniffing or checking, but only indifference follows. In contrast, younger subadult males proffer the same frequency of sniffs to frontalin but significantly more pre-flehmen and flehmen responses, and often vocalize or exhibit a variety of retreat

Table 9 First test comparisons: pregnant female Asian elephants compared with follicular- and luteal-phase females [values are medians (25–75 percentiles)]

Response category	Responses/hour			
	Pregnant	Luteal phase	Follicular phase	SSD
MO	1.00 (1.00–1.50)	1.50 (0.00–2.00)	3.50 (3.00–4.00)	follicular, $P = 0.05$
Pre-flehmen	7.00 (4.50–10.00)	0 (0.00–0.00)	5.50 (5.00–6.00)	luteal, $P = 0.005$
Flehmen[a]	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.50 (0.00–1.00)	none

SSD = statistically significant difference.

[a] No significant difference, $P = 0.09$.

Table 10 Repetitive tests: three pregnant female Asian elephants (P_4 = 462–1426 pg/ml)

Female	No.	Month into pregnancy	Responses/hour to 100 μM frontalin		
			MO	Pre-flehmen	Flehmen
E	2	12	1	4	0
E	3	14	0	2	0
E	4	15	0	3	0
Range			0–1	2–4	0
Average			0.33	3.0	0
Median			0.00 (0.00–0.75)	3.00 (2.25–3.75)	0.00 (0.00–0.00)
L	1	9	1	12	0
L	2	20	1	3	0
L	3	20	1	6	0
Range			1	3–12	0
Ave			1	7.0	0
Median			1.00 (1.00–1.00)	6.00 (3.75–10.50)	0.00 (0.00–0.00)
Y	2	2	9	12	1
Y	3	2	10	12	1
Y	4	6	6	20	0
Range			6–10	12–20	0–1
Average			8.3	14.6	0.66
Median			9.00 (6.75–9.75)	12.00 (12.00–18.00)	0.00 (0.00–0.00)

Table 11 Frontalin-evoked chemosensory responses and sequential behaviors: female Asian elephants in three hormonal states: follicular phase, luteal phase and pregnant

State	Female	Chemosensory response	Behavior	Additional sequential behavior	*n*
Luteal	E	sniff →	avoid	trumpet	1
	S	sniff →	avoid	circle the sample	2
Pregnant	L (9 months)	sniff →		rumble, urinate, palatal pit →	1
	K (1 month)	sniff →	rumble →	palatal pit to L	1
	K (1 month)	sniff →	4 palatal pits to L →	palatal pit to self	1
	E (2 months)		palatal pit to female T		2
	Y (2 months)	sniff →	rumble		3
	Y (2 months)	check →	rumble → mouth gape	long time at sample	1
		sniff → 4 palatal pit to B →	checked temporal gland of B		1
	Y (6 months)		backup to → self palatal pit		1
Follicular	B	check → place →	checked male penis →	checked male temporal gland	1
	B	check → place → scrub →	flehmen → long time at sample	6 palatal pit to Y	1
	S	flehmen →	check male temporal gland		1

n = number of observations.

postures (Table 3). Females also exhibit varied immediate responses that segregate according to their individual hormonal condition and reproductive state. Luteal-phase females, while not disinterested in frontalin, have a much lower response than follicular-phase females in all categories of chemosensory responses. While the olfactory responses of luteal-phase and pregnant females were similar in frequency, pregnant females were very responsive in the pre-flehmen category, exhibiting many check, place and suck responses, enabling identification of frontalin and probably its concentration.

These olfactory/vomeronasal organ responses were reinforced by sequential behaviors that suggested differential functionalities for frontalin. Subsequent or simultaneous behavioral responses, occurring after observed chemosensory responses to frontalin, were consistent within groups of similar hormonal phases (females) or maturity status (males). For example, luteal-phase females, after sniffing frontalin, exhibited immediate avoidance behavior (i.e. circling the frontalin sample) (Table 11). Pregnant females, with a higher frequency of sniffing, frequently rumbled after touching frontalin, whereas the checking of a conspecific's temporal gland after responding to frontalin suggests an attempt to identify the source of the chemical signal. In contrast, follicular-phase females performed numerous pre-flehmen and flehmen responses, at times followed by checking of the male penis or temporal gland, with no evidence of aversion.

The sexual and hormonal status differences in response to frontalin are consistent with the different lifestyles of males and females. The male lifestyle involves a more independent existence, greater wandering, and more curiosity and boldness in sampling new odors and tastes (Sukumar, 1989). In this study, young males demonstrated the highest responsivity, especially related to vomeronasal olfaction, and were more overtly dramatic in their avoidance or repulsion to frontalin than either highest responding females (those in the follicular phase or pregnant), or older experienced males who were indifferent to moda musth secretions (Rasmussen *et al.*, 2002) and somewhat indifferent to frontalin. In the tightly knit female society, females track each other's state of estrus (Slade, 1999; Slade *et al.*, 2003). Their responses to urine and urogenital secretions are influenced by their hormonal status (Rasmussen and Schulte, 1998; Schulte and Rasmussen, 1999). After encountering frontalin (or older male TGS), follicular-phase females respond with chemosensory responses and subsequently with reciprocal palatal pit responses and vocalizations. Such behaviors suggest inter-female transfer of information.

The two procedures for bioassays provide robust comparative data sets for defining frontalin as a pheromone of Asian elephants. For both sexes, bioassay data demonstrated that first tests (Tables 1, 4, 8 and 9) and repetitive testing (Tables 2, 5 and 10) gave similar results. Single tests are often preferred in behavioral studies to avoid pseudoreplication problems. However, to establish that a particular compound is a robust pheromone, it is necessary to demonstrate that elicited responses are not novel substance reactions. Repetitive testing of the same individuals confirmed the robustness of frontalin as a putative pheromone.

In examining pheromonal actions in elephants, their high cognitive abilities and excellent long-term memories, especially regarding odors (Rasmussen, 1995, 1999b), need to be considered. For a meaningful study of captive male elephants, a personal life experience history, including past and present musth status, hormonal levels, and past associations that account for individual differences, is necessary to discern possible functions. For example, one male OA1, although of similar age to the other older adult males, is subdominant and is less socially mature as the result of his particular life experiences; consequently, his responses for the first two assays fit the pattern of a younger male. As the dominance dynamics of the males changed, his responses fit into the adult grouping.

Comparison of the targets for musth male-released frontalin with that of the urinary-derived female sex pheromone (*Z*)-7-dodecenyl acetate demonstrates a clear difference in specificity of response (Rasmussen *et al.*, 1996, 1997; Rasmussen, 1999a). (*Z*)-7-Dodecenyl acetate elicits responses specifically from male elephants, whereas frontalin elicits varying behaviors and chemosensory responses from both males and females. Such a profile of directed behavioral responses to the production of sex-specific compounds has been observed in other mammals. For example, male mice release 3,4-dehydro-*exo*-brevicomin (an oxygenated terpene related to frontalin) in their urine that elicits male aggression and female attraction (Novotny *et al.*, 1985; Jemiolo *et al.*, 1991). This effect is further enhanced by co-secretion of farnesene (Novotny *et al.*, 1990). In the future, we will examine whether any of the substituted cyclohexanones (Perrin and Rasmussen, 1994) in TGS potentiate frontalin-elicited behaviors and determine whether several identified pheromonal ligand carriers (Rasmussen *et al.*, 1998, 2001; Lazar, 2001; Lazar *et al.*, 2001, 2002) have roles in effectively modulating these additional compounds in the ecological milieu of the Asian elephant.

It is interesting to speculate on the remarkable coincidence that frontalin, *Z*-7-dodecenyl acetate, and dehydro-exo-brevicomin—all previously identified as pheromone constituents in insects—also function in strikingly similar roles in higher organisms. Most likely it is a consequence of the comparatively limited biosynthetic capability of animals to produce volatile compounds, with lipid and terpene synthons (Francke *et al.*, 1995) being obvious choices for small molecular weight pheromonal ligands. Of interest therefore is the source of frontalin in elephants. Is it synthesized by males *de novo* or, more likely, is it transformed from plant-derived terpenic precursors by microbial action in the fermentative hind gut of the elephant and transported in the blood for release in the TGS? Evidence for the latter scenario comes from the finding of frontalin in the blood of musth males (L.E.L. Rasmussen, unpublished results). It will also be interesting to determine the chiral form of elephant frontalin. Both (+) and (–) forms may be active since the racemate supplied in the bioassays in the current study is obviously functioning in responsive individuals.

In conclusion, we have demonstrated that the bicyclic ketal frontalin functions as a male-generated pheromone in the Asian elephant, eliciting a range of behaviors in conspecifics depending on their sex, age and physiological state.

Acknowledgements

We thank Gary Jacobson and Jim Williams at the RCEC, Scott and Heidi Riddle at the Riddle's Elephant Sanctuary, and Roger Henneous, formerly of the Oregon Zoo, for their assistance in the bioassays. Biospherics Research Corporation and a travel grant from RCEC made this study possible.

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Accepted April 30, 2003