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Induction of Estrus in Grouped Female Mice (*Mus domesticus*) by Synthetic Analogues of Preputial Gland Constituents

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Abstract

Two major volatile constituents of the male mouse preputial gland, E,E-α-farnesene and E-β-farnesene, were examined for their role in inducing estrous cycles in grouped female mice. The results indicated that the mixture of the farnesenes was as effective as the homogenate of the intact preputial gland, while the extract of the castrate preputial tissue did not show a pronounced response.

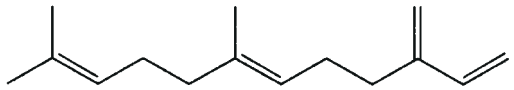
Introduction

Primer pheromones play a critical role in the reproductive physiology of the house mouse (*Mus domesticus*) and other rodents (Whitten and Champlin, 1972; Bronson, 1974, 1979; Bronson and MacMillan, 1983). Since the 1960s, urine has been investigated as the main source of the primer pheromones that are chiefly responsible for estrus synchrony (Whitten, 1956), puberty acceleration (Vandenbergh, 1969), puberty delay (Drickamer *et al.*, 1978) and pregnancy block (Bruce, 1959). The subsequent chemical characterization of some of these pheromones, together with biological testing of their synthetic analogues (Novotny *et al.*, 1985a, b; Jemiolo *et al.*, 1986; Jemiolo and Novotny, 1993; M. Novotny *et al.*, submitted for publication), has laid a foundation for a better understanding of the physiological and biochemical processes related to chemical communication in *M. domesticus*.

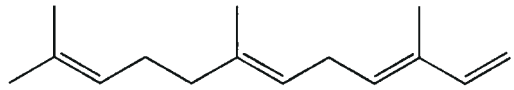
Estrous cycles of the house mouse are closely related to caging conditions. While isolated females exhibit 4–5 day cycles, grouped females have been reported to develop longer and irregular cycles (Whitten, 1959). Induction of estrus in crowded females is one of the best documented pheromonal phenomena. When the grouped females are exposed to a male, or male urine, the majority are stimulated into estrus with a high percentage occurring on the third day (Whitten, 1956). The male pheromones are androgen-dependent, as castration depresses the estrus acceleration capacity (Bruce, 1965). Since male urine alone was capable of inducing estrus in grouped female mice (Marsden and Bronson, 1964; Bronson and Whitten, 1968), the active substances must originate from either the internal metabolic products (originating from the liver or kidney metabolism) or, alternatively, from the products of some androgen dependent sex accessary glands which are located in the path of voided urine. However, there has been much confusion concerning the source of the male chemosignals. Already in 1968, Bronson and Whitten (1968) reported that the androgen-dependent primer pheromone for estrus induction was present in the bladder urine. Since the bladder urine was found as potent as the voided urine in its biological effect, the authors concluded that the preputial gland was not the source of the pheromone. In contrast, other investigators (Gaunt, 1968; Chipman and Albrecht, 1974) traced the biologically active (estrus-accelerating) substances to the preputial gland. A more recent paper (Marchlewska-Koj *et al.*, 1990) also implicates this gland as an important source of the estrus-inducing chemosignal.

The development and metabolism of the preputial gland is androgen-dependent (Burdick and Gamon, 1941). The preputial glands were reported as the source of androgen-dependent olfactory stimuli, such as the aggression-promoting signal (Mugford and Nowell, 1971) or a sex attraction pheromone (Bronson and Caroom, 1971). Based on these results, and the fact that individual molecules can function in both releaser and primer activities (for a review see Novotny *et al.*, 1990a), the preputial gland is a reasonable candidate for a source of estrus induction signal.

Since the earlier behavioral studies implicating preputial gland secretions to aggression (Mugford and Nowell, 1971) and sexual attraction (Bronson and Caroom, 1971), some progress has been made in chemical analysis of these secretions. E,E-α-farnesene and E-β-farnesene (Figure 1) are two major constituents (accounting for nearly 80% of its volatiles) of the male mouse preputial gland secretions (Novotny *et al.*, 1990b) excreted into urine. The farnesenes



E-β-Farnesene (M.W. 204)



E,E-α-Farnesene (M.W. 204)

Figure 1 Chemical structure of E,E-α- and E-β-farnesenes.

are odoriferous, terpene-like substances which occur in other natural sources: trail markers of red fire ants (Van der Meer *et al.*, 1981), alarm pheromones of aphids (Bowers *et al.*, 1972) and even a defense substance of wild potato plants against the aphids (Gibson and Pickett, 1983). The farnesenes were also found in Mediterranean fruit flies (Baker *et al.*, 1985) and the dorsal gland of the springbok (Berger *et al.*, 1981).

The concentration of farnesenes in male mouse preputial glands depends on the endocrine and dominance status of animals (Novotny *et al.*, 1990b). While castrates have lower levels of farnesenes than intact males, dominant males have higher concentrations than subordinate animals (Harvey *et al.*, 1989). Both dominant and subordinate males avoid marking areas soiled with stimulus samples containing either natural or synthetic farnesenes (Jemiolo *et al.*, 1992). The farnesenes also are attractive to female mice (Jemiolo *et al.*, 1991).

Following a partial elucidation of the farnesenes' role as behaviorally active chemosignals (releasing pheromones; Harvey *et al.*, 1989; Novotny *et al.*, 1990b; Jemiolo *et al.*, 1991, 1992), we are now investigating their potential primer functions. The purpose of this work was to examine if farnesenes are the active components of the preputial gland in estrus induction in grouped females. The effect of synthetic farnesenes was evaluated along with preputial gland homogenates from either intact or castrated male mice.

Materials and methods

Sexually experienced female mice (*M. domesticus*) of the ICR/Alb strain (Harlan Sprague Dawley Inc., Indianapolis, IN), which were ~3.5 months old and exhibited normal 4–5 day estrous cycles, were used for the experiments. The animals were maintained under standard laboratory conditions (22°C; relative humidity, 60–70%). A 12 h light: 12 h dark daily regime was followed, with lights on at 06.00 h. Formulated food (Purina Mills, Richmond, IN) and water were supplied *ad libitum*. The wood shavings used for bedding were changed weekly.

Two sets of experiments were conducted using densities of four (set I) and eight animals per cage (set II). The dimensions of cages were 12 × 27 × 17 cm. Prior to the exposure to olfactory stimuli, the animals were grouped together for 3 weeks to cause a degree of estrus suppression (Whitten, 1959). Four treatment groups were employed for each set of experiments: (i) distilled water (serving as a control); (ii) an aqueous extract of preputial glands from intact males; (iii) an aqueous extract of preputial glands from castrated males; and (iv) an equal-part mixture of α- and β-farnesene dissolved in distilled water at 250 p.p.m. (total concentration). Preputial glands collected from 40 intact and 39 castrated males of 4 months of age were homogenized with 40 and 39 ml saline solution respectively. (Castration was performed 1 month before the mice were sacrificed.) The homogenates were centrifuged (8500 **g** for 10 min) and their respective supernatants were used for treatments. The mixture of α- and β-farnesene was synthesized in our laboratory as follows: commercially available *trans*-nerolidol (Aldrich, Milwaukee, WI) was heated in hexamethylphosphoramide at 80°C for 12 h to yield a mixture containing equal amounts of E,E-α-farnesene and E-β-farnesene with a purity of >95%, as judged by its analyses by the combined gas chromatography/mass spectrometry and proton nuclear magnetic resonance spectrometry. One drop (~50 μl) of each stimulus sample was delivered directly to the nasal groove and external nares of the tested animals (held by hand) via a small plastic tube connected to a microsyringe. Each animal was treated twice daily for 10 consecutive days, first in the morning (08.00–10.00 h), and then in the afternoon (16.00–17.00 h). After each treatment, animals were immediately returned to the original cage. The estrous status of each female mouse was determined each morning for 10 days using the standard vaginal smear method (Rugh, 1990). A smear with cornified cells suggested estrus, while the appearance of persistent leukocytes and/or mucus in the smear indicated diestrus. All animals were individually labeled for identification. Animals exhibiting estrus for two consecutive days were not recounted. Each animal treatment group was housed in a separate testing room free of males. In each treatment group, 4–5 cages of animals (16–20 animals for set I, 40 for set II) were used. All animals were tested just once.

The number of animals exhibiting estrus in the first 5 days of treatments and the mean number of estrous cycles during the whole 10 day experimental period were recorded and considered as indicators for estrus synchronization and induction through the tested samples. The data were analyzed using χ^2 analysis and two-way analysis of variance (ANOVA) (Zar, 1984). The probability level for a significant difference was set at 0.05.

Table 1 Number of animals exhibiting estrus on the first 5 days of treatment

Treatment group	Number of animals	Day of treatment					Total number	Percentage
		1	2	3	4	5		
Set I: 4 mice/cage								
Control (water)	16	1	0	2	3	2	8	50.0
Preputial extract from castrated males	16	1	1	2	2	3	9	56.3
Preputial extract from intact males	20	1	4	5	3	5	18	90.0*
Solution of synthetic farnesenes	20	2	3	5	6	2	18	90.0*
Set II: 8 mice/cage								
Control (water)	40	5	4	3	3	3	18	45.0
Preputial extract from castrated males	40	2	4	6	5	4	21	52.5
Preputial extract from intact males	40	4	6	14	5	2	31	77.5*
Solution of synthetic farnesenes	40	4	7	9	9	3	32	80.0*

*Significantly different from control and castrated male groups (χ^2 test, $P < 0.05$).

Results

Table 1 contains the data for estrus disposition during the first 5 days of experimental treatments, showing the influence of various stimuli on estrus induction and synchronization. It is clearly demonstrated here that a significantly higher percentage of females ($P < 0.01$) treated with the aqueous extract of intact preputial glands or the solution containing synthetic farnesenes attained their estrus as compared with the animals treated with the extract from castrates or the water control. However, no significant difference was detected between the control and the extract of castrate preputial glands ($P > 0.05$), verifying that the biological activity of the preputial gland is under gonadal control. Since the levels of farnesenes are androgen-dependent (Harvey *et al.*, 1989), it is easy to explain why the castrate preputial gland is biologically ineffective. There was a characteristic days 3 and 4 peak of estrus (Marsden and Bronson, 1964) in the case of positive stimuli ($P < 0.01$). For example, in set II, the total occurrence of estrus on days 3 and 4 for the intact preputial group and the farnesene group was 47.5% (19/40) and 45% (18/40) respectively, while no such 'peak' was evident for the remaining two groups (15 and 27.5% for the water control and the castrate preputial gland respectively).

Comparing the results from sets I and II, it can be seen that the percentage of females stimulated into estrus appears to be higher for the less crowded environment ($P = 0.12$), though the difference did not reach significance at $P = 0.05$ under the current experimental design. It has been known that the degree of estrus suppression in grouped females is density-dependent (Champlin, 1971). Consequently, the effect of estrus induction by the odor stimuli is likely to be related to density. Thus, in a high-density situation, a reversal of estrus suppression by a positive male stimulus could be partially counterbalanced by a signal of female origin (Whitten, 1959; Ma *et al.*, 1998) or some other stress-related factors caused by overcrowding.

Table 2 Mean number of estrous cycles over the entire experimental period[1]

Treatment group	Set I (4 mice/cage)	Set II (8 mice/cage)	*P*
Control (water)	1.13 ± 0.26	0.90 ± 0.20	
Preputial extract from castrated males	1.25 ± 0.26	1.08 ± 0.20	
Preputial extract from intact males	2.05 ± 0.23*	1.73 ± 0.20*	0.07
Solution of synthetic farnesenes	2.10 ± 0.23*	1.75 ± 0.20*	

[1]Cages were used as statistical sampling units.

*Significantly different from control and castrated groups (two-way ANOVA, $P < 0.05$).

Table 2 summarizes the data for frequency of estrous cycles during the whole 10-day experimental period. The data support long-term inductive effects of the stimuli on estrous cycles. In both density conditions, the intact preputial extract and synthetic farnesenes caused significantly more estrous cycles ($P < 0.01$), than the control (water) stimulus and the extract of castrate preputial glands. Between the two density sets, there was some tendency of the animals from the four-per-cage condition to exhibit more frequent cycles, although the differences were not significant ($P = 0.07$). Once again, female-originated inhibitory chemosignals produced by crowding may exert some counterbalance to the stimulatory effect of tested samples.

Discussion

The results shown here verify the earlier reports (Gaunt,

1968; Chipman and Albrecht, 1974; Marchlewska-Koj *et al.*, 1990) on the effectiveness of preputial gland secretions in estrus induction. The estrus induction phenomenon is clearly androgen-dependent, since this capacity disappears after castration. The major androgen-dependent volatile constituents of the preputial gland secretion, the sequiterpenes α- and β-farnesene, seem to account for most observed biological activity associated with this gland.

We have previously demonstrated that farnesenes have some behavioral roles. They are the chemosignals causing territorial avoidance among male mice (Novotny *et al.*, 1990b; Jemiolo *et al.*, 1992) and attracting females (Jemiolo *et al.*, 1991). Here, we find their function as primer pheromones. It is likely that these two compounds act as multipurpose pheromones: signaling pheromones for an immediate behavioral control and primer pheromones for a physiological regulation. This does not seem unreasonable, as we have seen previously a somewhat parallel situation with dehydro-*exo*-brevicomin and 2-(*sec*-butyl)-4,5-dihydrothiazole, two compounds that are male-originated and androgen-dependent chemosignals. When added to castrate urine, these two compounds can act synergistically to potentiate inter-male aggression (Novotny *et al.*, 1985a), be attractive to females (Jemiolo *et al.*, 1985) and induce estrus synchronization (Jemiolo *et al.*, 1986). We have previously observed (Jemiolo *et al.*, 1991) that the investigatory preference for synthetic farnesenes was stronger for sexually experienced females than naive females. Sexual experience by females may thus also be important in the process of estrus induction. Nevertheless, estrous cycles of grouped virgin females were shown to be successfully induced by the stimuli from male mice (Marchlewska-Koj *et al.*, 1990; Marsden and Bronson, 1964).

Both bladder urine (Bronson and Whitten, 1968) and preputial glands [as shown here and elsewhere (Gaunt, 1968; Chipman and Albrecht, 1974; Marchlewska-Koj *et al.*, 1990)] contain chemosignals that are active in estrus induction. This suggests that estrus induction signals can come from multiple sources. Chipman and Albrecht (1974) pointed out that in addition to the urinary factor, a preputial substance may become incorporated into the male void urine and thus enhance the stimulation of estrus. Our previous study demonstrated that two bladder urine trace components, dehydro-*exo*-brevicomin and 2-(*sec*-butyl)-4,5-dihydrothiazole, potentiate the Whitten effect (Jemiolo *et al.*, 1986). The results of this work reveal that the two urinary farnesenes (produced in the preputial gland and excreted into the urine) are also active in estrus induction. At the molecular level, this supports the notion of the additive nature of chemosignaling from two metabolically distinct sources.

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