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Promotion of the Whitten effect in female mice by synthetic analogs of male urinary constituents

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ABSTRACT **Two volatile constituents of male mouse urine, 2-(*sec*-butyl)-4,5-dihydrothiazole and dehydro-*exo*-brevicomin, were synthesized and tested for their ability to induce estrous cycle in female mice (the Whitten effect). The suppression of ovarian cycling activity that resulted from grouping the females was abolished by exposure to normal male urine. The synthetic compounds, when added together in appropriate concentrations to the (previously inactive) urine of castrated males, or even to water, were found to be as effective as normal male urine. The action of the synthetic compounds appears attenuated for singly caged females.**

Stimuli from the social environment (primer pheromones) have been shown to influence both the frequency and composition of the estrous cycle in the mouse *Mus musculus* (1–4). Induction and synchronization of estrus among unisexually grouped females in the presence of a male [the Whitten effect (1)] and the male-induced failure of implantation and return to the estrous cycle [the Bruce effect (5)] are among the best known examples of male-to-female pheromonal effects in mice. Conversely, an all-female environment tends to suppress the estrous cycle: the effects of female grouping vary from an extension of the cycle length (6, 7), or prolongation of the quiescent phase of the estrous cycle (8), to induction of spontaneous pseudopregnancies (9, 10).

The pheromone(s) involved in the Whitten effect are excreted in the urine of intact adult males and their production is androgen-dependent (11). The active urinary substance(s) can elicit a variety of endocrine responses in the female mice (12–14). These male-originated substances appear to act with a high degree of specificity in altering the secretory patterns of luteinizing hormone and prolactin and of the steroids whose secretion is regulated by these two tropic hormones (15). Regulation of the ovulatory function is among the most important reproductive consequences of these hormonal processes.

Although it is becoming increasingly clear that both small and large molecules can act as mammalian pheromones (16–19), the results of Whitten *et al.* (20) and Gangrade and Dominic (21) support the idea that an airborne (volatile) signal is responsible for the Whitten effect. Analytical methods are now sufficiently advanced to elucidate structural features of the molecules causing various pheromonal effects.

During comprehensive studies of the volatile components of urine of *Mus musculus* (22), two structurally unique substances have been found in association with the intact male: 2-(*sec*-butyl)-4,5-dihydrothiazole [2-(*sec*-butyl)thiazoline] and dehydro-*exo*-brevicomin. Through organic synthetic efforts (23), adequate quantities of these substances are now available for biological testing. In parallel with possible pheromonal activities, the urinary concentration of dehydro-*exo*-brevicomin is drastically reduced following castration, but testosterone supplementation restores it to the normal level (24).

As we recently found that both dehydro-*exo*-brevicomin and 2-(*sec*-butyl)thiazoline act as intermale aggression stimuli (25) and attractants to females (26), the present investigations were designed to evaluate the possible role of these compounds in the regulation of female fertility. Consequently, these synthetic chemosignals were tested as dilute solutions in both male castrate urine and plain water. Their effect on the estrous cycle of individually or group-caged females has been examined against normal male urine (a standard stimulus) and water (control).

MATERIALS AND METHODS

The test animals were adult C57BL/6J virgin females, laboratory-bred, obtained originally from The Jackson Laboratory. The females were housed under standard laboratory conditions and maintained with 12 hr of light (0600–1800) and 12 hr of darkness daily.

The females (8–10 weeks old at the start of the experiments) were placed in equal numbers into three housing conditions: one, four, and eight females per cage (12 × 28 × 17 cm). In some experiments, females were housed at 10 per cage. Following the 28 days allowed for adaptation, the animals were exposed to the test stimuli samples, or to water as a control. A Petri dish (5 cm in diameter) containing a 0.25-ml sample aliquot was placed in the center of the cage housing the females. During the exposure period, the Petri dish with sample was placed in a wire-mesh hemicycle (5 cm in diameter and 2 cm in height) that prevented physical contact of the females with the sample but allowed exposure to the airborne olfactory cues. The animals were exposed to various olfactory stimuli daily for 15 min (between 1000 and 1200 each morning) for 21 days.

Each day, prior to exposure (between 0800 and 1000), all females were examined for their stage of vaginal cycle. Estrous cycle was classified using criteria of Vandenbergh (27) and Rugh (28). Groups of females were subjected to no more than two treatments, and a minimum of 3 weeks was always maintained between such tests.

Urine samples to be used as stimuli were collected from 12 normal and 12 castrated C57BL/6J males, aged 4–6 months (29), in metabolism cages. Urine samples were collected on a block of dry ice. Dehydro-*exo*-brevicomin and 2-(*sec*-butyl)thiazoline were mixed into castrate urine at concentrations simulating their content in normal urine (roughly 1.3 ppm, vol/vol)

Vaginal smears were examined from females housed during 21 days with 15 min daily exposure to (*i*) water (control), (*ii*) water plus 2-(*sec*-butyl)thiazoline and dehydro-*exo*-brevicomin, (*iii*) castrate urine, (*iv*) castrate urine plus 2-(*sec*-butyl)thiazoline and dehydro-*exo*-brevicomin, or (*v*) urine of

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Table 1. Mean number of estrous cycles in female mice housed at different densities and exposed to various stimuli

Stimulus	No. of estrous cycles per female		
	1 per cage	4 per cage	8 per cage
Water (control)	$3.1 \pm 0.3^{a,x}$	$2.7 \pm 0.2^{a,x}$	$1.6 \pm 0.3^{a,y}$
Castrated males' urine (CMU)	$3.2 \pm 0.3^{a,x}$	$2.6 \pm 0.2^{a,x}$	$1.5 \pm 0.3^{a,y}$
Intact males' urine	4.2 ± 0.3^{b}	3.9 ± 0.2^{b}	3.5 ± 0.3^{b}
CMU plus 2-(*sec*-butyl)thiazoline and dehydro-*exo*-brevicomin	3.4 ± 0.3^{a}	3.5 ± 0.2^{b}	3.3 ± 0.2^{b}

Values are the mean number (±SEM) of estrous cycles, during the period 3–21 days after initial exposure to stimulus, in female mice housed at 1, 4, or 8 per cage ($n = 8$ females per group). Those means in the same vertical column not marked with the same superscript letter (a or b) are significantly different at the 0.02 level, for the first column, and the 0.01 level, for the second and third column; the means in the same horizontal row not marked with the same superscript letter (x or y) are significantly different at the 0.01 level; if there are no superscript letters in a row (or column), there are no significant differences among the means.

intact males. Vaginal smears obtained from each test female were assessed under a microscope.

RESULTS

Effects of Grouping and Different Stimuli on the Length and Number of Estrous Cycles. Statistical analysis [Duncan's new multiple-range test (30)] of data presented on Tables 1, 2, and 4 showed that housing conditions and different stimuli samples had a significant effect on the number and lengths of estrous cycles during the 21-day experimental period. The estrous-cycle frequency of the control group (water) decreased with increasing cage population, only slightly for four animals per cage, but significantly for eight animals per cage (Table 1). For each cage-population density, the number of estrous cycles observed in females exposed to urine from castrated males was similar to that observed in the control group. Females exposed to the urine of intact males had similar numbers of estrous cycles, calculated from days 3–21 of treatment, at all population densities, and these figures were significantly higher (Table 1) than the corresponding values for females exposed to water or to castrate urine. The same effect was observed for females housed at four or eight per cage and exposed to dehydro-*exo*-brevicomin and 2-(*sec*-butyl)thiazoline added to castrate urine.

The decrease in cycle frequency that accompanies grouping is closely paralleled by an increase in the relative length of the anestrous stage. All females housed at eight per cage and exposed to water (control) or to castrate urine had significantly longer estrous cycles than did the females exposed to the same stimuli but housed at one or four per cage (Table 2). Females exposed to urine of intact males showed a similar length of cycle during treatment under all housing conditions. In addition, the castrate urine containing the volatile synthetic compounds induced, similarly, a high level of estrous cycling in the grouped females (Table 2).

Exposure to normal urine or to castrate urine mixed with the synthetic compounds produced estrus on the third or fourth day in the majority of females, whether they were housed individually or in groups (Table 3).

Data from a control group clearly indicate that grouping the C57BL/6J females at eight per cage results in lengthening the estrous cycle (Table 2). However, females housed in groups (four or eight per cage) in the presence of urine from intact males, or of castrate urine plus dehydro-*exo*-brevicomin and 2-(*sec*-butyl)thiazoline, exhibited shorter and more frequent cycles.

Effect of Urinary Chemosignals on the Estrous Cycle Synchronization in Females Housed at High Density. During the adaptation period, vaginal smears from females that were housed at 10 animals per cage were taken from the 7th to the 28th day (prestudy interval) of grouping. This provided an additional point of comparison for the average of complete estrous cycles in each experimental and control group. The

Table 2. Mean length of estrous cycles in female mice housed at different densities and exposed to various stimuli

Stimulus	Length of estrous cycle, days		
	1 per cage	4 per cage	8 per cage
Water (control)	$4.7 \pm 0.2^{a,x}$	5.2 ± 0.8^{x}	$8.2 \pm 0.3^{a,y}$
Castrated males' urine (CMU)	$4.6 \pm 0.2^{a,b,x}$	5.5 ± 0.8^{x}	$7.7 \pm 0.3^{a,y}$
Intact males' urine	3.9 ± 0.1^{b}	4.0 ± 0.7	4.6 ± 0.3^{b}
CMU plus 2-(*sec*-butyl)thiazoline and dehydro-*exo*-brevicomin	$4.6 \pm 0.2^{a,b}$	4.4 ± 0.8	4.5 ± 0.3^{b}

Values are the mean length (±SEM) of estrous cycles, during the period 3–21 days after initial exposure to stimulus, in female mice housed at 1, 4, or 8 per cage ($n = 8$ females per group). Those means in the same vertical column or horizontal row not marked with the same superscript letter (a or b, for columns, or x or y, for rows) are significantly different at the 0.01 level; if there are no superscript letters in a row (or column), there are no significant differences among the means.

Table 3. Percentage of female mice with estrus smears on days 3 or 4 after initial exposure to stimulus

Stimulus	% with estrus smears			
	1 per cage	4 per cage	8 per cage	10 per cage
Water (control)	38	50	25	20 (20)*
Castrated males' urine (CMU)	50	50	38	30 (25)
Intact males' urine	88	100	88	70 (30)
CMU plus 2-(*sec*-butyl)thiazoline and dehydro-*exo*-brevicomin	88	75	63	70 (20)
Water plus 2-(*sec*-butyl)thiazoline and dehydro-*exo*-brevicomin	ND	ND	ND	45 (30)

Number per group: $n = 8$ females for 1, 4, and 8 per cage; $n = 20$ females for 10 per cage. ND, not done.
*Values in parentheses give percentage of females with estrus smears in the prestudy intervals.

Table 4. Mean number and length of estrous cycles in female mice housed 10 per cage, before and after exposure to various stimuli

Stimulus	Number of estrous cycles		Length of estrous cycles, days	
	Prestudy*	During exposure†	Prestudy*	During exposure†
Water (control)	1.4 ± 0.2	1.4 ± 0.2[a]	7.7 ± 0.4	7.8 ± 0.4[a]
Castrated males' urine (CMU)	1.5 ± 0.1	1.6 ± 0.2[a]	7.8 ± 0.3	8.0 ± 0.3[a]
Intact males' urine	1.5 ± 0.2[x]	3.1 ± 0.2[b,y]	7.8 ± 0.3[x]	4.5 ± 0.2[b,y]
CMU plus 2-(*sec*-butyl)thiazoline and dehydro-*exo*-brevicomin	1.3 ± 0.1[x]	2.8 ± 0.2[b,y]	8.3 ± 0.5[x]	5.0 ± 0.2[b,y]
Water plus 2-(*sec*-butyl)thiazoline and dehydro-*exo*-brevicomin	1.5 ± 0.3[x]	2.7 ± 0.2[b,y]	8.3 ± 0.5[x]	4.5 ± 0.2[b,y]

Those means in the same vertical column or horizontal row not marked with the same superscript letters (a or b, for columns, or x or y, for rows) are significantly different at the 0.01 level; if there are no superscript letters in a row (or column), there are no significant differences among the means. $n = 20$ females per group.
*Days 7–28 of 28-day adaptation period.
†Days 3–21 after initial exposure to stimulus.

female mice living under crowded conditions (10 per cage) were then exposed to the stimuli samples twice per day for 15 min (between 1000 and 1200 and between 1700 and 1800 each day).

The effect of housing 10 females per cage is similar to that of housing 8 females together (Tables 1 and 2). The number and length of complete estrous cycles in females housed at 10 per cage, during 21-day prestudy intervals, were similar in all five treatment groups (Table 4). However, the suppressive effect of high population density was abolished (Table 4) by the exposure of animals either to normal male urine, or to castrate urine plus the synthetic compounds, or to water to which these synthetic compounds were added. However, water containing these compounds apparently was not sufficient to induce estrus in the females on the third or fourth day after exposure, whereas either normal male urine or castrate urine containing the synthetic compounds was sufficient (Table 3).

DISCUSSION

Suppression of the ovarian-cycle activity in group-caged adult female mice has been observed in different laboratories (8, 31, 32). In addition, various studies (11, 33–35) showed that such caging conditions may also lead to anestrus, but that the presence of a male or male's odor tends to synchronize estrus in the female animals. It is also well-documented that exposure of unisexually housed female mice to conspecific males will renew the estrous cycle in the majority of the females and will synchronize their estrus 3 or 4 days later. Females exposed to males or to their urine will exhibit shorter and more regular cycles than females kept in the absence of male stimuli (1, 20). However, no specific chemicals (pheromones) responsible for these effects were identified.

The results of Whitten *et al.* (20), Monder *et al.* (36), and Gangrade and Dominic (21) all suggested that the male signal involved in estrus induction was volatile. Since this is in contrast to the seemingly nonvolatile nature of another male mouse primer pheromone, the puberty-accelerating signal (16, 17), controversies abound. The studies reported here, including the experimental arrangement, show that the estrus-inducing male signal is indeed volatile.

Bronson and Macmillan (15) suggested that, under different conditions of perception, the same chemosignal may cause different biological responses in the recipient. We have verified this notion with two synthetic mouse-pheromone candidates, 2-(*sec*-butyl)dihydrothiazole and dehydro-*exo*-brevicomin; we have previously shown that these chemicals, when present in the overall context of male urine, will cause intermale aggression (25) as well as attract females (26). Yet another biological role of these compounds has become clear from the present study: their ability to induce the Whitten effect and to maintain a regular estrous cycle in grouped females closely parallels the effectiveness of the natural stimulus, normal male urine. However, their potency as a synchronizer was attenuated for the females not living in a group. This indicates that the effectiveness of these synthetic compounds is dependent on the reproductive status of females. The grouping of females in the absence of a male tends to prolong anestrous cycles or produce pseudopregnancies (10). Perhaps only in these states, which are endocrinologically similar to pregnancy, these synthetic chemosignals may cause synchronization of estrous cycles.

This report shows that synthetic chemicals can act as primer pheromones in a mammalian system. It is interesting that, unlike in behavioral assays related to aggression (25) and odor preference (26), the pure chemicals dissolved in plain water induce the Whitten effect. Evidently, our observations do not fit the common belief that mammalian signals (unlike insect pheromones) always involve a complex array(s) of natural substances.

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